UNIVERSITY OF ALBERTA

BACTERIAL INTERACTIONS WITH ANTHRACENE IN A MODEL SOIL SYSTEM

by

Anthony Neumann ©

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Bacterial Interactions with Anthracene in a Model Soil System submitted by Anthony Trent Neumann in partial fulfillment of the requirements for the degree of Master of Science in Microbiology and Biotechnology.

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DEDICATION

I dedicate this thesis to my dear wife Catherine who gave me the love and encouragement I needed at all times during the time I took to obtain this degree. You are the greatest wife anyone could ask for - I'm glad I got you before anyone else had the opportunity to find this out!!

I also dedicate this thesis to God who gave meaning to my life, opened doors for me to obtain this degree and gave me the opportunity to meet many new friends.

ABSTRACT

Due to the carcinogenicity of polycyclic aromatic hydrocarbons (PAHs), their removal from the environment is necessary. A model soil, glass beads, was used to monitor interactions that affected the rate of degradation of anthracene in a roller drum bioreactor.

The spontaneous dissolution rate of anthracene was suspected to affect the rate of biodegradation. The solubility of anthracene was dependent on the surface area of the anthracene and the quality of the surface that was exposed to the medium.

Two pure cultures were isolated from an enrichment culture. One slow growing Gram positive, acid-fast isolate, *Rhodococcus* S1, was known to metabolize anthracene. A 4 - 5 day lag period before degradation began was due to colonization of anthracene crystals. A fast growing, Gram negative isolate, *Pseudomonas* G1, was unable to utilize anthracene.

In coculture experiments grown on anthracene, *Pseudomonas* G1 inhibited the ability of *Rhodococcus* S1 to degrade anthracene. *Pseudomonas* G1 required the presence of *Rhodococcus* S1 in order to grow. In the presence of glycerol, this interaction was not observed. *Pseudomonas* G1 cometabolized a microbially produced phenanthrene dihydrodiol similar to that produced by *Rhodococcus* S1.

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LIST OF ABBREVIATIONS

ABBREVIATION	DEFINITION
PAH	Polycyclic aromatic hydrocarbon
GC	Gas chromatography
HPLC	High pressure liquid chromatography
TLC	Thin layer chromatography
SEM	Scanning electron microscopy
SOM	Soil organic matter
SATP	Standard ambient temperature and pressure
NAPL	Non aqueous phase liquid
cfu/mL	colony forming units per milliliter
K_{ow}	Octanol-water partition coefficient

1. INTRODUCTION

1.1 Remediation of petroleum based compounds

Polycyclic aromatic hydrocarbons (PAHs) have been released into the air, water and soil of most environments due to fossil fuel burning, leakage from commercial equipment, accidental spillage, improper storage or natural sources (Heitkamp and Cerniglia, 1987). Many of these compounds accumulate in the environment at a faster rate than nature can remove them.

Due to increasing knowledge of the toxicity of these contaminants, especially to humans, governmental standards for cleaner environments are becoming more stringent. Consequently, many remedial procedures to treat these contaminated ecosystems have recently been developed. Several of these procedures can remove PAHs from water very efficiently based on the insolubility of these compounds in water. However, soils and sediments tend to adsorb hydrophobic compounds making the removal of these compounds difficult.

As a result, many soil remediation techniques have involved the use of organisms found in the contaminated soil which are capable of lowering the toxicity by removing these contaminants. Biological treatment of contaminated sites is gaining popular appeal because of the abilities of soil microbial communities to transform many toxic chemicals into water and carbon dioxide. It was discovered nearly 70 years ago that some of the smaller components of PAHs could be degraded by bacteria in soil. Since that time, biochemical pathways and enzymes responsible for degradation of these hydrocarbons have become well understood.

This review will focus on (i) the chemical characteristics of PAHs; (ii) bacterial degradation of PAHs and (iii) how the soil environment affects the interactions of PAHs with the bacterial community. It will further discuss the importance of bacterial interactions

in soils contaminated with PAHs and PAH containing wastes such as creosote. Finally, the rationale for research done for this thesis will be presented.

1.2 Polycyclic aromatic hydrocarbons - definition

Polycyclic aromatic hydrocarbons are a group of organic compounds with two or more fused homocyclic condensed aromatic ring structures. Due to their aromaticity, PAHs are quite stable. They constitute a large class of chemicals of about 150 individual compounds (Sims and Overcash, 1983). Those PAHs with 3 rings or fewer are referred to as small PAHs while those with more than 3 rings are called large PAHs. Aromatic structures containing nitrogen, oxygen or sulfur are known as heterocylic aromatic compounds.

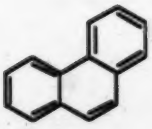
1.2.1 Physicochemical characteristics of polycyclic aromatic hydrocarbons.

Polycyclic aromatic hydrocarbons are generally characterized as being high melting, high boiling point solids with low vapour pressure at standard ambient temperature and pressure (SATP)(Raddig et al., 1976). Of all PAHs, only naphthalene and methylnaphthalenes are significantly volatile at SATP (Park et al., 1990). Aqueous solubility of PAHs decreases with increasing size and decreasing condensation of the structure. For example, anthracene and phenanthrene have the same number of aromatic rings but phenanthrene is more soluble than anthracene because it is more condensed than anthracene (see Fig.1.1). Similarily, condensed PAHs are more stable in the environment than uncondensed PAHs (Blumer, 1976). Polycyclic aromatic hydrocarbons dissolve readily in hydrophobic solvents and therefore have high n-octanol/water partition coefficients (K_{ow}). This signifies that they are extremely hydrophobic and when ingested or absorbed through the skin, will traverse the cell membrane and accumulate in fatty tissues. Therefore, they may biomagnify along a food chain.

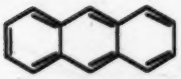
Figure 1.1 Polycyclic aromatic hydrocarbons listed as priority pollutants by the Canadian Environmental Protection Agency (except chrysene) and found in significant quantities in environments near industrial sources.

naphthalene

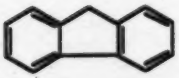
acenaphthene



phenanthrene

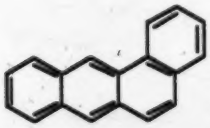


anthracene

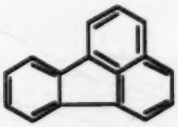


fluorene

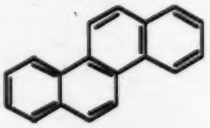
pyrene



benz[a]anthracene

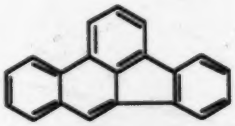


fluoranthene

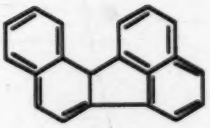


chrysene

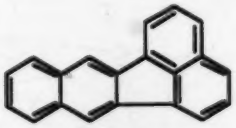
benzo[a]pyrene



benzo[b]fluoranthene



benzo[j]fluoranthene



benzo[k]fluoranthene

indeno[1,2,3-cd]pyrene

1.2.2 Production of PAHs

Production of PAHs, either through natural or anthropogenic means, occurs through a process known as pyrolysis. At very high temperature or high pressure and in a chemically reduced atmosphere (similar to temperatures prevailing at the center of a flame or pressures deep in the earth's subsurface) aromatic ring systems are the most stable organic chemical species while alkyl side chains are less stable. As the aromatic rings accumulate under these conditions, they polymerize into more complex structures. Alkylation of these polymers decreases as the heat increases (Blumer, 1976).

Depending upon the insufficiency of oxygen in this environment and the heat produced in the process, a wide variety of alkylated and non-alkylated compounds can be formed (Smith, 1984; Watt et al., 1984). If the heat produced is similar to that found in a forest fire, pyrolysis of plant constituents results in the production of PAHs with short alkyl side chains. On the other hand, low temperature/high pressure pyrolysis occurs in oil deposits and tends to produce more alkylated PAHs. In these conditions, the length of the side chains would be longer than those produced from forest fires (Blumer, 1976). Many PAHs found in petroleum are further pyrolyzed into more complex polymers due to incomplete combustion of fossil fuels by industry and automobiles (Grimmer et al., 1981) which leads to pollution of the air in highly populated or industrial areas (Daisey et al., 1979).

1.2.3 Sources and prevalence of PAHs

Polycyclic aromatic hydrocarbons are widely distributed throughout the environment and tend to persist for long periods of time (Shuttleworth and Cerniglia, 1996). The largest single source of PAH emissions into the environment in Canada is due to forest fires - approximately 2000 tonnes/yr (Canadian Environmental Protection Act, CEPA, 1994). However, forest fires cause a single large exposure to one specific area. It

has been shown that more extensive contamination of the environment will occur through the continuous emissions of PAHs from a single source.

The largest continuous anthropogenic source of PAHs in Canada arises from aluminum smelter plants utilising the Horizontal Stud Soderberg Process (CEPA, 1994) and close behind this are wood preservation facilitites. Both processes require liquid coal tar creosote, a source rich in PAHs. Creosote is predominantly composed of PAHs (≥85%) with smaller quantities of phenolics (10%) and heterocyclic compounds (Mueller et al., 1989). Creosote has, until recently, also been used as a pesticide (CEPA, 1994).

Other less concentrated industrial sources of PAHs have been found in soots, tars, sewage sludge, waste waters, tobacco smoke (International Agency for Research on Cancer, 1983), smoked foods (McGill et al., 1982; Lawrence and Weber, 1984), cooking oils (Lawrence and Weber, 1984b), grilled meat products (Larsson et al., 1983; Chen et al., 1996) and plants and vegetables grown near an industrial source of PAHs (Larsson and Sahlberg, 1982). Vegetables grown near urban centers accumulate 10 fold higher levels of PAHs than those grown in rural areas (Wagrowski and Hites, 1997). These plants absorb PAHs from the atmosphere and concentrate the contaminant in actively growing tissues (Suess, 1976). Uncommon (and still controversial) sources of PAHs arise from biosynthesis in organisms such as higher plants (Sims and Overcash, 1983), bacteria and freshwater algae (Andelman and Suess, 1970). However, PAHs in these natural sources are formed in small quantities compared to anthropogenic sources (Suess, 1976).

1.2.4 Contamination of the environment by PAHs

As of April 1993, there were 160 bioremediation projects in the USA which were monitored by the US Environmental Protection Agency (EPA)(Stringfellow and Aitken, 1994). Over half of these sites were contaminated with wood preserving wastes or petroleum. Traditional wood preserving techniques employ a 5% solution of pentachlorophenol (PCP), an uncoupler of oxidative phosphorylation, as a biocide in

creosote. Once the liquid creosote has been applied to the wood, the logs are left to absorb the creosote resulting in complete saturation of the pores of the wood. Due to the procedure, a portion of the treatment solution is inevitably deposited in the soil, rendering it hydrophobic and toxic. Many of the pore spaces in the soil become filled with PCP and creosote. If the soil particles have a high organic content, the creosote binds very rapidly to the soil, making it difficult to remove by any physical mechanism. As will be discussed later, the saturation of these pore spaces reduces the number of microsites available for active microbially enhanced degradation.

Atmospheric deposition of PAH particulate matter due to incineration processes has been proposed to be the main source of PAHs found in most soils and sediments (Christensen and Zhang, 1993). High temperature PAH vapors cool rapidly and condense onto particles in the air or form particles of pure condensate (Strand and Andren, 1979). Due to their hydrophobicity, PAHs will also adsorb strongly to suspended solids in the water column (Watt et al., 1984). Once they are adsorbed to the particle, the rate of desorption is very slow (Pignatello and Xing, 1995). As a result, PAHs that are adsorbed to atmospheric dust will eventually be deposited either in water (and eventually into sediments) or in soil by wind or precipitation (van Noort and Wondergem, 1985). Because they adsorb so strongly to particles in the water, about 90% of all PAHs in the water environment can, theoretically, be removed by sedimentation, sand filtration and/or activated charcoal treatment (Watt et al., 1984; Middaugh et al., 1991).

Levels of PAHs in the environment have been increasing since the Industrial Revolution began in the 19th century. A sediment core retrieved from a bay near high levels of industrial activity was analysed for the distribution of PAHs. Estimating the rate of deposition of the sediments in this bay, Hites et al., (1977) analysed the settling of PAH particles over the last 100-150 years and found that the levels of PAHs in the sediments had increased 10 fold. They compared this to sources of energy used in the last century and were able to develop a simple model showing that the increased combustion of fossil fuels

due to industry and automobiles are responsible for much of this increase in PAH pollution. Similarily, Simcik et al. (1996) analysed sediments in a lake located near coking plants and aluminum plants and discovered the increases of PAHs in the sediments mirrored increases in emissions by these industries.

1.2.5 Toxicity of PAHs

In 1775, Potts was the first to find that an environmental cause, a component of chimney soot, was the cause of scrotal cancer in chimney sweeps. Over 100 years later, scientists finally isolated the infamous PAH in soot responsible for causing cancer - benzo(a)pyrene (Sims and Overcash, 1983).

Many of the high molecular weight PAHs (≥ 4 rings) are known to be genotoxic and carcinogenic. Benzo[a]pyrene is the most carcinogenic PAH known and, as a result, much research has focussed on the removal of this compound from the environment. The three ring PAHs are suspected to be carcinogenic and are on the EPA priority pollutant list. Currently 13 PAHs are on the list of priority pollutants by CEPA (1994) (Fig. 1.1). Naphthalene, the smallest PAH, is not known to be carcinogenic; however, it has an acute toxicity effect in mammals where large doses of naphthalene can cause hemolytic anemia. Interestingly, early and current uses of naphthalene include moth repellent (it is the odor of "moth balls"), and intestinal or topical antiseptics (Merck, 1976).

Unsubstituted PAHs are biologically inert (Sims and Overcash, 1983) but they tend to accumulate in hydrophobic areas of the cell such as lipids and intercalate between DNA basepairs. This, in itself, does not seem to make the PAHs mutagenic. However, metabolism of the PAH to form an epoxide by the action of mammalian microsomal monooxygenases increases the mutagenic properties of these compounds (Ames and Grover, 1972). Ames and Grover (1972) have proposed that the epoxides of PAHs may intercalate into and covalently bind to DNA, causing frameshift mutations. Frameshift

mutagens alter the reading frame of DNA so that transcription into RNA results in the translation of faulty proteins.

PAHs are of considerable concern in the aquatic environment because of their ability to accumulate in freshwater invertebrates (Southworth et al., 1978). In fact, the more insoluble the PAH, the higher the bioaccumulation potential. The PAHs tend to accumulate in the lipid fraction of organisms. An accurate estimate of the bioaccumulation potential of PAHs can be found using the K_{ow} - the higher the partition coefficient the greater the accumulation potential. Lee et al. (1978) have also found that the ability of oysters to remove PAHs from tissues decreases with increasing size of the PAH. Thus, large PAHs such as benzo(a)pyrene accumulate very quickly in aquatic invertebrates and are removed quite slowly. This creates the problem of bioaccumulation of large PAHs in the food chain.

Additionally, Muoz and Tarazona (1993) have found that combinations of PAHs synergistically affect the toxicity of these compounds to water invertebrates.

1.3 Degradation of PAHs

1.3.1 Bacterial studies

Many organisms, both eukaryotic and prokaryotic, can degrade PAHs but only fungi and bacteria are capable of mineralizing PAHs. Enrichment of cultures of bacteria capable of using PAHs as sole carbon and energy sources began fifty years ago, when Sisler and ZoBell (1947) discovered mixed bacterial cultures of marine origin which were capable of mineralizing a variety of PAHs including benzo(a)anthracene. Since that time an active search for pure cultures capable of degrading these carcinogenic compounds has continued.

Microorganisms capable of degrading PAHs have been isolated from a wide variety of ecosystems. Because of the ubiquitous distribution of PAHs, most environments have bacteria capable of using such compounds as sole carbon and energy sources. However,

Herbes (1981) and others (Herbes and Schwall, 1978; Lee et al., 1978) have shown that large continuous inputs of PAHs into the environment stimulate communities to degrade fractions of the contamination faster than communities from a pristine environment. It may be that the enzymes responsible for degrading such pollutants are dependent on exposure of the microorganism to levels of pollutant high enough to induce the production of these enzymes (Bauer and Capone, 1985; Heitkamp and Cerniglia, 1987; Heitkamp et al., 1987). As a result these contaminated environments are effectively enrichment cultures for a variety of PAH degraders.

Therefore, most pure cultures of PAH degraders are isolated from contaminated sources. Many of these organisms can be grown in simple medium containing nitrogen, phosphate and trace metals required for growth. Usually, a single PAH is added as the only carbon and energy source. Some isolates require more complex nutrients to stimulate growth on some PAHs. From this isolation procedure, several genera of bacteria have been shown to metabolise PAHs. These include *Pseudomonas* (Evans et al., 1965), *Flavobacterium* (McKenna, 1976), *Achromobacter* (Cutright and Lee, 1995), *Sphingomonas* (Ye et al., 1996), *Mycobacterium* (Heitkamp and Cerniglia, 1989; Boldrin et al., 1993), *Rhodococcus* (Tongpim and Pickard, 1996), *Alcaligenes* (Weissenfels et al., 1990), *Micrococcus* (Ashok et al., 1995), and *Beijerinckia* (Mahaffey et al, 1988) to mention a few.

1.3.2 Removal of PAHs from the environment

There are several ways in which PAHs are degraded in the environment. One mechanism through which they can be removed from the environment is volatilization. This process, though, is negligible in soil at temperatures below 80°C except for the simplest of PAHs - naphthalene and methylnaphthalenes (Park et al., 1987).

The aromatic ring structure of PAHs causes them to be very stable and unreactive (Sims and Overcash, 1983). However, PAHs can be chemically activated by the addition

of molecular oxygen. Two methods of activation exist: photooxidation and biological oxidation. In the atmosphere and the upper layers of water environments, photoxidation predominates (Suess, 1976). However, in soil, sediments and wastewater, biological degradation is the main mode of removal of PAHs (Shuttleworth and Cerniglia, 1996). Other minor modes of abiotic degradation of PAHs include ozonation (Fouillet et al., 1991; Chen et al., 1979) and chlorination of PAH-polluted water at water treatment plants. Chlorination of PAHs and humic substances can result in the formation of trihalomethanes in drinking water (Norwood et al., 1980). These compounds are strong mutagens and can be difficult to remove in an aerobic environment. Few aerobic microorganisms have the enzymatic capability to remove chlorine from an aromatic structure, resulting in increased persistence of these compounds in the environment.

1.3.2.1 Abiotic removal of PAHs

Many processes release PAHs into the atmosphere as aerosols which tend to bind to particles in the air. Depending on the size of the particle, they can persist in the air from less than 1 day to 6 weeks (National Academy of Sciences, NAS, 1972). Precipitation decreases the residence time of these particles in the air (van Noort and Wondergem, 1985) whereas wind and convectional air currents increase the residence time. Photodecomposition of PAHs in the atmosphere occurs through reactions with a variety of oxidants in the atmosphere, such as nitrogen oxides, sulfur oxides and ozone (NAS, 1972).

The rate of photodecomposition of PAHs in water, either adsorbed to particles or in aqueous solution, increases with increasing oxygen concentration, temperature, and illumination (Andelman and Suess, 1971). Therefore compounds nearest to the surface of the water will degrade faster than those deeper in the water. Consequently, PAHs which were not photooxidized settle into the sediments of lakes and rivers where abiotic processes occur very slowly due to reduced illumination and oxygen concentrations (Suess, 1976).

Sediments effectively act as a sink for PAH accumulation due to the low oxygen concentrations which also makes biological removal slow.

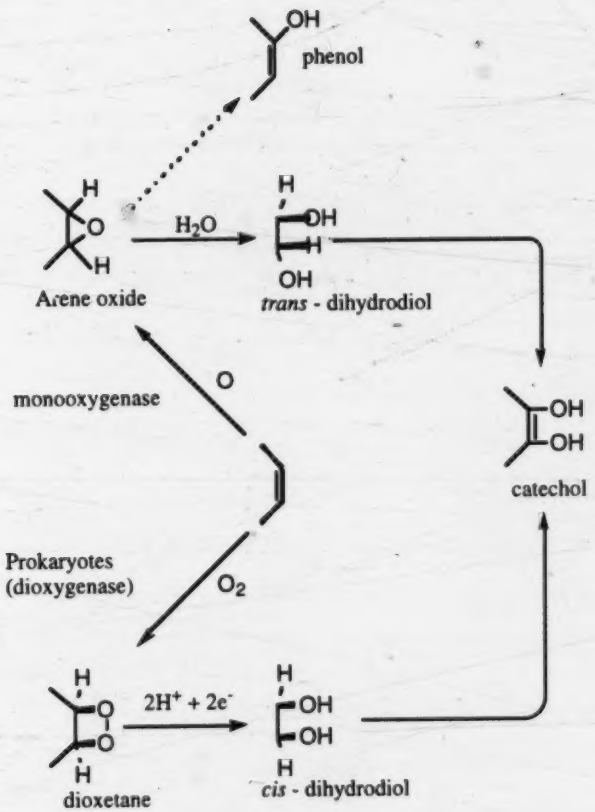
1.3.2.2 Biological removal of PAHs

Once PAHs enter the soil or sediments, degradation is primarily biological. They can be bioaccumulated and partially metabolized in plants and animals or they can be completely mineralized by bacteria and fungi in the soil or sediment. Because PAHs are biologically inert and insoluble in water they must be activated and solubilized by the addition of molecular oxygen (Hayaishi and Nozaki, 1969). Therefore, rapid degradation of PAHs occurs only in aerobic environments. This activation is accomplished through enzymes called oxygenases, of which two classes exist: monooxygenases and dioxygenases (Gibson, 1968).

Monooxygenases are found in both prokaryotes and eukaryotes. These enzymes catalyse the addition of a single atom of oxygen into the substrate and form a reactive intermediate called an arene oxide. This reactive substrate is further capable of spontaneously reacting with water to form the vicinal *trans* dihydrodiol. However, due to the electrophilicity of the epoxide intermediate, the arene oxide is also capable of covalently reacting with other biological compounds such as DNA which can result in frameshift mutations (Ames et al., 1972) and tumor biogenesis (Gibson, 1976).

All known dioxygenases are produced exclusively by bacteria and catalyze the incorporation of two atoms of oxygen into the substrate (Gibson et al., 1975). As a result, the compound formed is a dioxetane which is not as electrophilic as the arene oxide formed by monooxygenases. The dioxetane bond is reduced and the *cis* isomer of the dihydrodiol is formed. From this point, both the *trans* and the *cis* isomers of the dihydrodiol are oxidized to the same corresponding catechol. The cascades of enzymatic events initiated by monooxygenases and dioxygenases are compared in Figure 1.2.

Figure 1.2 Comparison of two enzymatic activations of aromatic compounds. Oxidation of an aromatic ring by monoxygenase and dioxygenase enzyme systems. Formation of the arene oxide by monooxygenases can cause frameshift mutations in DNA (Sims and Overcash, 1983; Cerniglia et al., 1979).


OH
phenol
H
O
H
H_2O
H
OH
H
OH
Arene oxide
trans - dihydrodiol
monooxygenase
O
OH
OH
catechol
Prokaryotes
(dioxygenase)
O_2
H
O
O
H
$2H^+ + 2e^-$
H
OH
OH
H
dioxetane
cis - dihydrodiol

Research by Rogoff and Wender (1957) and Colla et al. (1959) concentrated on the biochemistry of PAH metabolism by soil microorganisms. They found that phenanthrene and anthracene were attacked at the end ring to form the corresponding *cis* -dihydrodiol.

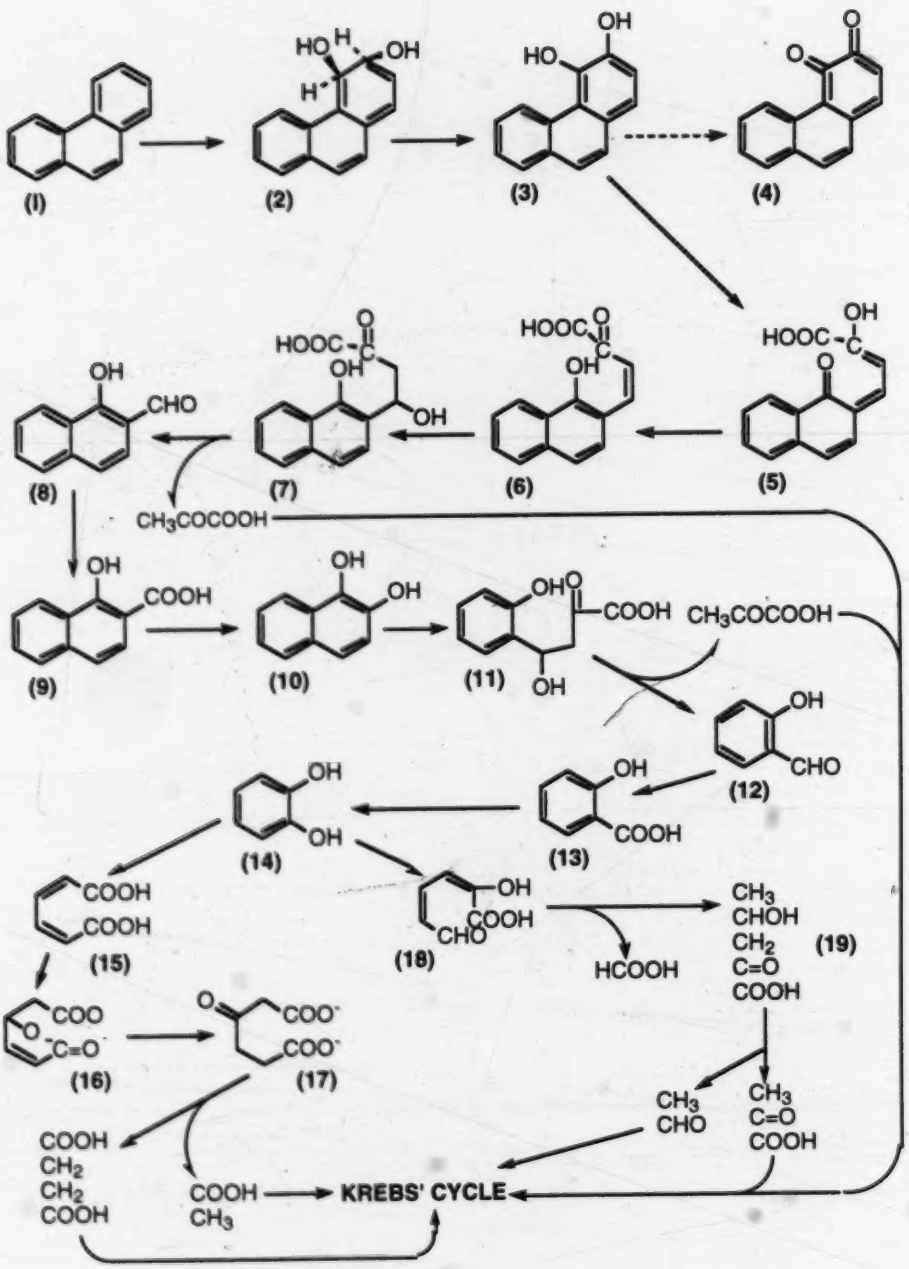
The ring fission mechanism of aromatic compounds can occur via two different mechanisms. Both of these pathways involve dioxygenases. The first of these, known as *ortho* fission, cleaves the carbon-carbon bond between the hydroxyl groups of the catechol intermediate (Hayaishi and Nozaki, 1969). The resulting compound is a *cis,cis* - muconic acid. The other mechanism of ring fission, *meta* fission, oxidizes the catechol to the alpha-hydroxymuconic semialdehyde by cleaving the bond beside the hydroxyl groups (Dagley et al., 1960). Evans et al. (1965) found that phenanthrene and anthracene ring fission products in soil pseudomonads arise predominantly by the latter mechanism. After fission of the first ring in these compounds, pyruvate and CO_2 are released. The cycle repeats itself with dihydroxynaphthalene (Figure 1.3) to form catechol, pyruvate and CO_2. The catechol ring is then broken into Krebs' cycle intermediates (Alexander, 1977) and mineralization of the PAH is complete.

The persistence of PAHs in the environment increases with increasing size of the PAH. As a consequence, there has been much research (and progress) following the mineralization of small PAHs while studies into the mineralization of large PAHs by pure cultures is not as prevalent in the literature. Until recently, no pure bacterial isolate from creosote contaminated sources was capable of mineralizing PAHs with more than 3 rings (Weissenfels et al., 1990). Large PAHs have been shown to be partially metabolized by inducible enzymes involved in similar pathways by adding inducing compounds such as salicylate to the culture medium (Mahaffey et al., 1988). This oxidation appears to follow the same trend as the metabolism of small PAHs except that the initial oxidation is not as specific and therefore multiple ring fission products have been found for compounds like benzo[a]pyrene (Schneider et al., 1996).

Figure 1.3 Mineralization of phenanthrene by a typical soil pseudomonad.

The phenanthrene ring is first activated by the addition of oxygen and then one ring is cleaved by either *ortho*- or *meta*- cleavage. The latter mechanism is shown in this pathway. The components of the ring are removed by cleavage of pyruvate side chains and the process is repeated for the remaining two rings.

The metabolites of the pathway are as follows: **(1)** phenanthrene; **(2)** *cis*-3,4-dihydro-3,4-dihydroxyphenanthrene; **(3)** 3,4-dihydroxyphenanthrene is spontaneously oxidized quickly in aqueous solution to **(4)** 3,4-phenanthoquinone, a dead end product; **(5)** *cis*-4-(1-oxonaphthyl)-3-hydroxybut-1,3-dienoic acid is an expected intermediate formed during transformation to **(6)** *cis*-4-(1-hydroxynaphth-2-yl)-2-oxobut-3-enoic acid. An aldolase attacks the side chain via intermediate **(7)** to release pyruvate and produce **(8)** 1-hydroxy-2-naphthaldehyde. An NAD-dependent dehydrogenase converts **(8)** to **(9)** 1-hydroxy-2-naphthoic acid which is decarboxylated to form **(10)** 1,2-dihydroxynaphthalene. Compound **(10)** is similar to **(3)** and the ring is cleaved by *meta* -cleavage to form **(11)** *cis*-o-hydroxybenzalpyruvic acid. Pyruvate is again released from the ring to produce **(12)** *o*-hydroxy-benzaldehyde. Compound **(12)** is transformed into **(13)** *cis*-o-hydroxybenzoic acid (salicylate) which is further processed to **(14)** catechol. The final ring is cleaved by either *ortho*-cleavage to form **(15)** *cis,cis*-muconic acid or by *meta* -cleavage to form **(18)** 2-hydroxymuconic semialdehyde. Compound **(15)** enters the Krebs' cycle by transformation of **(16)** muconolactone to **(17)** ß-oxoadipate and finally succinate and acetate. Compound **(18)** enters the Krebs' cycle by release of formate to form **(19)** 2-oxo-4-hydroxy valerate which are further processed to pyruvate and acetaldehyde (Sims and Overcash, 1983; Cerniglia, 1984; Alexander, 1977; Evans et al., 1965; Kiyohara and Nagao, 1978).


(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
(13)
(14)
(15)
(16)
(17)
(18)
(19)
CH3COCOOH
HCOOH
KREBS' CYCLE

Even so, these large PAHs tend to persist in the soil much longer than small PAHs. Cometabolic interactions between communities of soil microorganisms are thought to be an important mechanism leading to the degradation of these persistent compounds.

1.3.3 Cometabolism

Cometabolism has been defined as a process which "occurs when an organism growing on a particular substrate gratuitously oxidizes (metabolises) a second substrate it is unable to utilize as nutrient or energy source" (Atlas and Bartha, 1993). Cometabolic transformations occur when an organism growing on a certain compound produces the enzyme for the oxidation of that compound. This enzyme may have a broad substrate specificity and therefore may oxidize a cosubstrate as well. However, the next enzyme involved in the metabolism of the substrate may be much more specific and unable to further metabolise the cosubstrate resulting in a dead end product in pure cultures. This metabolite may serve as a possible carbon or energy source for another organism found in the mixed culture. This is one of the bases for a commensalistic relationship. These cometabolic transformations have been proposed as an important means by which many recalcitrant compounds are mineralized in soil communities although little research currently exists examining cometabolism of large PAHs.

Two types of cometabolism are currently recognized: analogue substrate enrichment and non-analogue substrate enrichment (Keck et al., 1989). Analogue enrichment utilizes a biodegradable compound that is structurally similar to the cometabolized substrate. Ideally, this results in the production of a broad specificity oxygenase capable of oxidizing the cometabolite. In our laboratory, a *Rhodococcus* sp. appearing to exhibit analogue enrichment has been isolated. Of a number of PAHs tested, this microorganism is capable of utilizing only anthracene and 2-methylanthracene as sole source of carbon and energy (Tongpim and Pickard, 1996). However, in the presence of anthracene (the analogue substrate) a large range of PAHs can be cometabolised to their corresponding diols

(Tongpim, 1997). This may suggest that the oxygenase responsible for ring oxidation has a very broad specificity while the dioxygenase responsible for ring cleavage is very specific and unable to use all other oxidised PAHs .

Non-analogue substrate enrichment occurs when an organism growing on one substrate oxidises a structurally different compound. A classic example of this was found by Beam and Perry (1974). *Mycobacterium vaccae* JOB5 was isolated for its ability to grow on propane as sole source of carbon and energy. When cyclohexane was added to this system, *M. vaccae* was capable of oxidising cyclohexane to the corresponding ketone which, in pure culture, was not further metabolised.

1.4 The soil environment

The heterogeneous environment of soil is very different from that found in traditional laboratory research. Bossert and Bartha (1984) compared soil environments to freshwater, marine and atmospheric environments and found that generally, "soil is the most complex portion of the biosphere characterized by intimately interwoven solid, liquid and gas phases. It has a wide range in sizes and a tremendous complexity in chemical composition." The complexity of soil has led Burns (1983) to define it as a "diverse community of microorganisms competing for fluctuating levels of unevenly distributed and frequently insoluble nutrients subject to fluctuations".

Soil can range in temperature from -40°C in the Antarctic valleys to 65°C in the hot sand of deserts. Water availability in the soil varies from air dried soil to waterlogged soil while the pH of soil ranges from acidic in mining ponds to highly alkaline soils like desert sand (Bossert and Bartha, 1984). Soil also has a much higher abundance of organic matter when compared to water environments and it has a greater number of attachment surfaces for both the contaminant and bacteria to adsorb onto. Soil approximates the work of a filter, able to prevent many highly lipophilic contaminants from entering the groundwater, provided the many binding sites found in the soil matrix do not become saturated.

1.4.1 Chemical properties of soil

Soil is composed of four distinct components: the mineral fraction, air/water phase, soil organic matter and biotic fractions. The quantity of each of these varies between each soil type. Generally, the mineral fraction of soil constitutes about 40-45% of the total volume and it is derived from the erosion of rock. The air/water phase, known as pore spaces, comprises about 50% of the total volume of soil. The organic portion of soil varies between 0.4% and 10% of the total volume and the biotic portion of soil usually comprises less than 1% of the total volume (Alexander, 1977).

The mineral portion of soil can be further divided into four fractions based on the size of the particle. The largest particles, gravel or stones, have a diameter that exceeds 2.0 mm. Sand is smaller with a diameter ranging between 0.05-2.0 mm. Silt ranges in size from 0.002 and 0.05 mm in diameter. The smallest of the mineral portions of soils are clays with diameters less than 0.002 mm.

Clay particles, due to their small size, have a much larger surface area than any of the larger components of soil. Since surface area is directly related to the chemical properties of the soil and is related to activities of bacteria, nutrients and organic matter in the soil, clays exhibit the most prominent role in determining the properties of soil (Alexander, 1977). Silt and sand exhibit a principal effect on the movement of water and exchange of air.

A substantial portion of soil is composed of pore spaces filled with water and air. The total pore space is dependent on the structure of soil. In clay soils, the pores are very small and numerous whereas in sandy soils, the pores are very large but less abundant. Accordingly, dry clays have a higher diffusivity than dry sandy soils and therefore have better gas exchange properties. However, when the clay becomes wet, water is retained more than in sandy soils and gas exchange in clays becomes poor.

Clay, silt and bacterial polysaccharides in soils tend to adhere together to form larger structural units known as aggregates. These are temporary, unstable structures which affect water and air exchange. Aggregates are important in the adsorption, diffusion and, thus, the bioavailability of PAHs to microorganisms surrounding or within the aggregate. Aggregates are also important from a microbiological perspective as they may have a completely different soil water environment when compared to the surrounding soil. For example, the pH in a microsite may vary by three pH units when compared to the surrounding bulk phase (McLaren and Skujins, 1968).

Most organic substrates entering the soil are either insoluble, or soluble but packaged in cells and tissues with insoluble barriers (Nedwell and Gray, 1987). The organic matter in soil arises from plant material, animal tissues and excretory products. When these are subjected to decay, simple soluble carbon compounds leach out from these sources and are quickly taken up and metabolised by the soil community. The larger insoluble compounds persist by becoming incorporated into the brown humus fraction of the soil. In turn, the microorganisms degrading these nutrients also serve as a source of carbon and energy for succeeding generations.

A diversity of heterogeneous substances are released during decomposition in the soil which microorganisms can assimilate. Many of the polymeric substances are degraded by the soil community into their monomeric constituents such as phenols, amino acids and sugars. Some of these compounds are quickly used by cells as a source of carbon and energy. However, some of the more recalcitrant compounds undergo spontaneous or enzyme-catalysed chemical reactions causing these compounds to react with lignin degradation products to form complex organic molecules known as humic, fulvic and humin substances (Atlas and Bartha, 1993). Amino acids, sugars, phenols, lipids and PAHs can form further linkages to the humic substances, thereby increasing their complexity and heterogeneity (Kästner et al., 1994). Humic compounds are in a constant flux with the continuous polymerization of organic constituents balanced with their rate of

mineralization. Mineralization is usually enhanced by slow growing compost degrading organisms.

Microorganisms make up a very small proportion of soil yet they execute a very important role in soil. They are responsible for the recycling of humus and other organic detritus in the carbon cycle, nitrogen fixation in the nitrogen cycle and they provide other nutrients necessary for plants and animals to grow. Microbial numbers and diversity in the upper portions of soil are generally much greater than found in air or water environments. Bacteria found in soil may be obligate aerobes, facultative anaerobes, aerotolerant, microaerophilic or obligate anaerobes. Many of the slow growing microorganisms use refractory substances such as humic acids as their source of carbon and energy while other opportunistic indigenous organisms exhibit rapid growth only upon the addition of easily utilizable sources including animal carcasses, droppings and contaminants.

Different groups of microorganisms will exhibit the dominant role in response to a change in the environment. Therefore, at any one time some organisms will be in a state of inactivity depending on the soil environment (pH, moisture content, etc.). For example, if the pH of the soil is acidic, fungi would recycle many complex bioavailable organic materials while activity of bacteria would be suppressed. On the other hand bacteria express the dominant activity in soils which are slightly alkaline (Sims and Overcash, 1983). Additionally, waterlogging the soil results in anaerobiosis of the pore spaces and activity by obligate aerobes would cease.

Due to the complexity of the soil composition, aggregates that are formed in clay soils may have very heterogeneous inner and outer environments. Aggregate formation may entrap both substrates and microorganisms effectively isolating them into their own microhabitats (Nedwell and Gray, 1987). Due to the different microenvironments, a completely distinct microbial community may exist within the aggregate when compared to those outside the aggregate. This tremendous diversity of organisms in soil and the many

microhabitats in which they are forced to exist, compel microorganisms to interact in order to survive in such a competitive ecosystem.

1.5 Bioavailability and biodegradation of PAHs in soil

Because of the complexity of the soil environment and the complex mixtures of PAHs in creosote and petroleum contaminated sites, there are many factors that may enhance or inhibit the availability of the compound in a particular soil. The most important factors that affect bioremediation of a polluted soil are the chemical characteristics of the soil and the PAH, the environmental conditions of the soil and the biological community present in the soil. The percentage of sand, silt, clay and organic matter often determine the way PAHs interact with a soil and thereby affect the community of microorganisms involved in degradation of the contaminant. At the same time, biodegradation of PAHs can be enhanced by maintaining oxygen concentration, water availability, temperature, pH, and nutrients near optimal levels (Wilson and Jones, 1993). Another important factor affecting bioavailability of the substrate to microorganisms is the composition of the creosote such as the proportion of large PAHs or biocidal components (PCP) in the mixture. Finally, the actual community of bacteria in the soil will affect both the rate and the degree of bioremediation.

1.5.1 Effects of PAH structure on degradation

The physical state of the PAH greatly influences bioavailability of the substrate to the bacteria (Volkering et al., 1992). The contaminant cannot be degraded if it is physically separated from the degrading organism. Most microbes appear to degrade the contaminant only if it is dissolved in aqueous solution (Wodzinski and Coyle, 1974). Solubility of PAHs decreases with increasing size and decreasing condensation (Bossert and Bartha, 1986). Furthermore, the rate of solubilization or desorption of PAHs determines their rate of biodegradation in liquid culture (Thomas et al., 1986; Stucki and Alexander, 1987).

Since larger, uncondensed PAHs are less soluble than small PAHs, they usually persist longer in the environment. Because of the low rates of solubilization of large PAHs, bacteria using these compounds as sources of carbon and energy tend to grow linearly in liquid culture rather than exponentially as is the case with soluble carbon sources (Volkering et al., 1992).

1.5.2 Soil characteristics affecting biodegradation

The composition of soil also plays a crucial role in the bioremediation of contaminated sites because it affects the bioavailability of the contaminant and it affects the adsorption characteristics of the soil for PAHs. The bioavailablility of PAHs can be decreased by partitioning of the PAH into components of the soil which bacteria may be unable to affect. Separation of the contaminant from the bacteria can occur in a variety of circumstances. Many pollutants within a highly contaminated site may exist in liquids known as nonaqueous phase liquids (NAPL) that are immiscible with water and are thereby sequestered from the microbial community (Alexander, 1994). Because bacteria require aqueous environments for life, the nearest they can come to the contaminant is to the water -NAPL interface and the rate of PAH metabolism is less when compared to similar levels of PAH contamination in the absence of NAPL (Efroymson and Alexander, 1994). The contaminant may also be physically separated from the degrading organism if it diffuses within a small soil pore making the organic contaminant inaccessible to larger bacteria.

1.5.2.1 Sorption of PAHs to the soil matrix

Adsorption of the contaminant to soil components also plays an important role in bioavailability. Adsorption decreases the bioavailability of the contaminant and thereby reduces the rates of biodegradation (Weissenfels et al., 1992).

Once PAHs enter any ecosystem they tend to adhere to particles (Poster et al., 1996). If these associations are formed in the air or water, they will eventually settle into

the soil or sediments. Meanwhile, coal tar creosote from wood preservation sites enters directly into the soil. Upon addition to the soil, the compound may then partition into the soil matrix and may enter the aqueous pore spaces.

Polycyclic aromatic hydrocarbons are continuously being adsorbed and desorbed from soil particles as they move through the soil column. Due to the hydrophobicity of PAHs, they tend to adsorb onto the soil longer than being desorbed in the soil water. The energy required for a PAH to desorb from a hydrophobic location increases with increasing size and decreasing solubility of the compound. Therefore, aqueous concentrations of PAHs in natural systems are dependent on adsorptive/desorptive equilibria within the soil (Dzombak and Luthy, 1984). On the positive side, adsorption of PAHs to the soil decreases their mobility and therefore prevents contamination of the groundwater. However, adsorption can decrease the availability of the contaminant to an organism capable of degrading it resulting in greater persistence of that compound in the environment.

Three factors in soil affect this adsoption/desorption equilibrium: the proportion of clay, the proportion of soil organic matter in the soil and the proportion of dissolved organic matter in the soil water. Clays, such as montmorillonite, are negatively charged and tightly bind cations. Adsorption of any compound to clays may involve van der Waals forces, hydrogen bonding or ion exchange (Alexander, 1994). If the electrolyte concentration is high enough in the clay, all anionic charges will be neutralized and organic neutral molecules may adsorb to the clay particle by van der Waal's forces (Yanagita, 1990). These are generally weak associations and PAHs easily desorb from such sites. If the compound is aminated, however, then the attraction may be stronger due to the cationic nature of the amine group (Subba-Rao and Alexander, 1982). Karimi-Loftabad et al. (1996) have also shown that dry clays may encourage polymerization of PAHs within a solvent upon addition to the soil (as would happen in terrestrial oil spills or wood

preservative industries). These polymerized PAHs would become much more recalcitrant and bind with stronger affinity to the soil matrix.

Tucker et al. (1995) discovered that the percentage of sand, silt and clay in a soil influences the degradation of pyrene by the white rot fungus, *Phanerochaete chrysosporium*. They found that non-sterile native soil was the most inhibitory to growth of this organism on pyrene followed by sterile native soil > clay > silt > sand. They hypothesized that the pyrene may have sorbed onto the clay and silt and became non-bioavailable to the extracellular enzymes released by this fungus.

1.5.2.2 Sorption of PAHs to soil organic matter

The soil organic matter (SOM) is also an important factor in reducing the bioavailability of hydrophobic neutral organic compounds like PAHs to the microbial community (Ogram et al., 1985). Dzombak and Luthy (1984) found that PAHs partition out of the aqueous phase into organic constituents of the soil and that the effects of sorption to the clay constituents are small in comparison. Some of these sorbed substrates become more resistant to biodegradation than non-sorbed material. Polycyclic aromatic hydrocarbons are extremely lipophilic and accordingly have high K_{ow} and tend to partition readily into SOM.

Manilal and Alexander (1991) set out to determine why biodegradation in soils and sediments was slower than liquid cultures in the lab. They found that the amount of phenanthrene that partitioned into the aqueous phase decreased in soils with increasing concentrations of SOM. All the phenanthrene added partitioned into the SOM in soils containing more than 30% organic material.

Other research has found that the addition of soil organic matter in the form of compost inhibits the degree of biodegradation because the PAHs and their metabolites bind tightly or irreversibly to the organic matrix in the compost making it non-bioavailable (Kästner et al., 1995; Kästner and Mahro, 1996; Richnow et al., 1993). Once

incorporated into the humus fraction the PAH constituents in the soil decreased rapidly suggesting that the degraders in compost are more accustomed to the breakdown of humus as carbon and energy sources.

Weissenfels et al. (1992) found that sand sorbed PAHs could be completely degraded by a mixed culture in a few days. However, addition of PAHs to soil with higher organic content resulted in a residual non-degradable fraction. The non-degradable fraction was extracted and added to the sandy soil and nearly complete degradation was observed. The extract was also added to the same soil with high SOM and elevated levels of degradation were seen followed by a plateau in the rate.

Similarily, Bossert et al. (1984) discovered that addition of an oily sludge onto soil by the "landfarming" technique resulted in mineralization of components of the sludge by the microbial community immediately after addition of sludge and undegraded hydrocarbons were then incorporated into the organic fraction of the soil.

Partition of the PAH into soil is a bimodal process. First, fast adsorption of PAHs occurs when they physically adsorb or partition onto hydrophobic areas of the soil surfaces. The thermodynamic driving force of PAHs to sorb to SOM is their mutual exclusion of water but the interaction usually occurs through weak dipolar forces (Pignatello and Xing, 1996). At this stage, the PAHs are still in contact with pore spaces and available to bacteria attached to surfaces in those pore spaces.

Slow adsorption/desorption processes then begin to take over. Upon entering the SOM, the PAH encounters a more ordered stagnant water film. Diffusion becomes slower. As time progresses, the compound may then enter fissures in the SOM too small for bacteria to enter. Diffusion becomes further retarded due to sorption and desorption of the PAH molecule to the sides of the pores. Eventually, the compound may enter micropores where steric hindrance will slow diffusion even more. Depending on the structure of the SOM, solid phase diffusion may also occur further reducing the bioavailability of the compound (Pignatello and Xing, 1996). This process may take weeks or months before

equilibrium is reached. Therefore, aging of the soil contaminated with PAHs prior to the addition of microbes reduces their bioavailability.

One factor that may increase the bioavailability and mobility of PAHs at a contaminated site is the dissolved organic carbon (DOC) in the aqueous portion of the pore spaces (Herbert et al., 1993). Maxin and Kögel-Knabner (1995) found that water extractable SOM may decrease the amount of large PAHs bound to soil by one order of magnitude. It appears that DOC may act quite similarly to surfactants added to the soil, by binding or surrounding hydrophobic contaminants in the soluble organic matrix of the DOC but it is uncertain whether this increased solubility actually increases the bioavailability of the compound.

1.5.3 Environmental factors affecting biodegradation

1.5.3.1 Oxygenation

Most current bioremediation processes depend on the presence of oxygen to catabolise PAHs. The aeration status of the soil depends on the total amount of air filled space in the soil, the size of the pore spaces, and the rate of oxygen consumption by the microbial community (Bossert and Bartha, 1984). For example, sandy soil, which has large pore spaces with a lot of air filled space permits a high rate of bacterial activity because air can diffuse into these spaces from the atmosphere. Microorganisms in sandy soil also have a low rate of oxygen consumption due to the presence of low quantites of organic matter. Therefore if an available carbon and energy source is added a favorable environment for biodegradation would be encountered.

On the other hand, clay soil has very small pore spaces and may result in poor diffusion of oxygen if the soil is wet. The introduction of carbon sources to this soil will increase the rate of oxygen consumption to a point where the soil will rapidly deplete its oxygen stores and become anoxic. Consequently, PAHs in the soil would no longer be rapidly catabolised by aerobic mechanisms.

The rate of oxygen diffusion decreases with increasing depth into the soil and the rate of biodegradation concomitantly decreases with decreasing oxygen tension (Freijer, 1996). Because of the low diffusion rates of oxygen into soil water, creosote contaminated soils are usually oxygenated in the first few centimeters only. Thus, most biodegradation occurs in this thin upper crust and any PAHs entering the anoxic environment below become very persistent unless an external source of oxygen is supplied by either tilling, aerating or adding hydrogen peroxide.

1.5.3.2 Moisture and temperature

Another important characteristic of the soil is the moisture content because moisture is essential for life. If all the pore spaces in the soil are completely saturated, then the soil is said to be waterlogged. Because of the poor diffusion rate and solubility of oxygen into the soil water, the soil can become anoxic very quickly. Conversely, if the soil is completely air dried (0.5 - 4% water) as in desert sand and there is no available free water for the microbial community, degradation of any organic matter will cease to occur. In the oil-contaminated Kuwaiti desert, where 22×10^6 barrels of oil spilled out over 7 months, degradation of oil by bacteria was most affected by the moisture. In the dry season, no degradation occurred. However, during the wet season, approximately 45% of the oil was degraded. Amendment of the sand with nitrogen sources further increased the rate of degradation (Radwan et al., 1995).

Dibble and Bartha (1979) found that the rate of degradation of an oily sludge by a microbial community in a sandy loam remained optimal between 30% and 80% of the water holding capacity of that soil. Levels above 80% or below 30% resulted in severe limitations to the rate of degradation.

Although it has not been examined extensively, temperature can play an important role in determining the rate of biodegradation of PAH contaminated sites, especially in the temperate Canadian climate where temperatures can vary from -40°C to 30°C throughout

the year. Predictably, Sexstone et al. (1978) found that biodegradation of oil ceases in frozen soil and the overall persistence of the contamination was very long in Artic soils. Bauer and Capone (1985) found that anthracene mineralization rates tripled in marine sediments when the temperature was changed from 10°C to 30°C.

Temperature seems to control the community structure of microorganisms in that soil environment. Westlake et al. (1974) showed that changing only the temperature at which northern crude oils were biodegraded altered the composition of the community of degraders and thereby the fraction of the crude oil which was degraded. Most soil organisms are mesophiles with an optimal temperature of 25°C and capacity to grow between 10°C and 45 °C. Maliszewska-Kordybach (1993) found that degradation of small soluble PAHs are more sensitive to temperature effects than large PAHs.

1.5.3.3 pH and nutritional requirements

The pH of soils can encompass a wide range although most are slightly acidic (Bossert and Bartha, 1984). Accordingly the pH of the soil can also alter the community of aromatic degraders. While many bacteria are sensitive to acidic conditions, fungi are more resistant. Consequently, fungal numbers increase and the bacterial numbers decline as the pH falls and fungi play a more prominent role in the bioremediation of acidic habitats (Alexander, 1977). Increasing the pH by the addition of lime would result in a complementary increase in bacterial numbers. Fungal degradation rates of PAHs are usually less than rates obtained by bacteria, therefore liming of the site usually results in an increase in overall PAH removal.

If all the above factors affecting biodegradation are within optimal parameters, then the addition of nitrogen and phosphate to the soil as fertilizer may further increase the rate of degradation. Sites that have been contaminated for an extended period of time may require these nutritional amendments and Venosa et al. (1996) found that in freshly contaminated beach plots, amendments that seeped into unammended sites from nearby

plots were sufficient to initiate degradation. It has been estimated that the C:N:P ratio in soils should be 100:10:1 for optimum rates of bioremediation to occur.

1.5.4 Mixed substrate effects on biodegradation

Soil environments not only vary according to soil composition and environmental factors but they also vary according to carbon and energy sources available to bacteria. The composition of the PAH contamination also affects the extent and rate of their degradation in soil. In creosote contaminated soil, PCP is present as a biocide and communities must overcome its toxic effects before degradation of the other components of creosote can occur. Similarily, naphthalene can have acute toxic effects on some bacteria, therefore, it must be removed before degradation of larger PAHs will occur (Bouchez et al., 1995). Communities of bacteria seem to have a larger capacity to overcome these toxic effects than pure cultures (Bouchez et al., 1995). In addition, it was found that two or three PAHs present in the sample may have synergistic toxic effects against small invertebrates (Muoz and Tarazona, 1993).

On the other hand, the presence of simple PAHs in the environment may stimulate production of dioxygenases which may also attack larger more persistent PAHs (Mahaffey et al., 1988). Acclimation of bacteria to single PAHs simultaneously acclimates the community for transformation of several different PAHs in marine sediments (Bauer and Capone, 1988). However, many different PAHs present in the contamination may inhibit the removal rates of a single PAH due to the competition for the same initial enzymes in the pathway for the metabolism of small PAHs (Stringfellow and Aitken, 1995).

1.5.5 Mixed culture effects on biodegradation of PAHs

There are many advantages to studying the mineralization of PAHs by pure cultures. The first of these is the isolation of metabolites and enzymes from the degradation process and understanding how an organism can obtain energy from degrading such a

contaminant. This has led to the complete pathway used by soil pseudomonads in the mineralization of small PAHs (Fig. 1.3). A second advantage to pure culture work is that one can optimize growth parameters for that isolate and determine organism-substrate interactions. By determining the nutritional requirements for a certain isolate to grow on the appropriate substrate a small degree of the biochemical interdependency within a community can be seen (Bull, 1980). But, there are several serious drawbacks in pure culture studies.

Pure cultures in any environment do not exist outside the laboratory and therefore biodegradative capacities of the community will be different than those observed in the laboratory. The biodegradative capacity of communities is quantitatively and qualitatively greater than pure cultures (Trzesicka-Mlynarz and Ward, 1994) as is the degree of resistance to toxic components in the community. Pure culture studies also discount the importance of genetic exchange within a community (Bull, 1980). Most large PAHs are thought to be degraded by a mixed community of bacteria where some nutrients necessary for the oxidation of the compound by the one bacterium are supplied by another organism or the cometabolic products are used as a source of carbon and energy by another organism in the mixed culture (Mueller et al., 1989). Mahaffey et al. (1988) found that benzo(a)anthracene was mineralized by a mixed culture upon induction by known metabolites of small PAHs.

The study of mixed cultures also brings the researcher one step closer to determining appropriate bioremediation strategies because it aids in the understanding of what biological factors are affecting bioremediation in that environment. It is not certain whether the pure cultures enriched on PAHs even play a prominent role in the microbial activity of the soil from which they were isolated because of the selectivity of most enrichment procedures.

Some research has gone into the development of a genetically engineered "super degrader". However, the degree of competition for nutrients in the soil environment may

be a reason why large inoculations of pure cultures capable of mineralizing a contaminant very rapidly in the laboratory rarely enhance degradation in the field even when abiotic factors have already been accounted for. Only Heitkamp and Cerniglia (1989) have found that competition with the indigenous population did not adversely affect the ability of a pyrene degrading *Mycobacterium sp.*. However, this organism was added to a pristine water environment to which PAH contamination was added at the same time. Thus, in such an environment, little competition for pyrene as a carbon and energy source exists. In soil, though, the microbial community is much more competitive, and in soil contaminated with PAHs for an extended period of time the indigenous population would already have evolved into a community capable of degrading these PAHs at a rate faster than that observed in uncontaminated environments.

Kästner et al. (1994) have found that PAH contaminated sites have a potent mineralizing community and that this community does not appear to be the limiting factor for the degradation of small PAHs. To date there have been no reports showing that allochthonous bacteria can increase the rate of biodegradation of PAHs in a contaminated soil environment. Therefore, an understanding of the interactions among mixed populations in these environments would be interesting and informative. Some examples exist. Li et al. (1996) isolated a two component culture which was capable of mineralizing pyrene very rapidly. However, upon separation of these two cultures, mineralization of pyrene ceased, showing the dependence of these two organisms to use pyrene as a sole carbon and energy source. Further research on the interaction of these organisms may have proven interesting.

Because mixed populations like this have a greater degradative capacity and a greater resistance to toxic components of the contaminantion, the search for a "super degrading mixed culture" might be more fruitful than genetically engineering an organism into a "super degrader".

1.5.5.1 Interactions within mixed cultures

When two organisms live within close proximity of each other in an ecosystem, such as a soil aggregate, they will probably interact. There are three possible ways one organism may interact with the other: favourably (+), unfavourably (-), or neutrally(0). Therefore, between two species there are nine interaction combinations: three cooperative interactions (0 +), (+ 0), (+ +); five offensive interactions (- -), (0 -), (- 0), (+ -), (-+) and one neutral interaction possibility (0 0) (Yanagita, 1990).

Mutualism (+ +) is an interaction where both species benefit from the association. In fact, they must interact with each other in order to survive. Very few examples of this can unequivocally be confirmed in contaminated soil communities because most often multiple carbon sources exist for utilization by the community. Only in anaerobic sewage sludge, where competition for carbon is great but energy yields are quite low, has mutualism been confirmed between methanogens and acetogens. In aerobic soil environments, if one organism cannot grow on a compound without help from another organism then it may also grow on another substrate in the soil.

Protocooperation (+ +) is also an interaction where both species benefit; however, they do not require the association in order to survive. Each organism supplying a nutrient that the other may be lacking in order to degrade a component of the contaminant would exemplify protocooperation. If the only carbon source is the contaminant in the soil then this relationship would become mutualistic. Dangmann et al. (1996) isolated a two component community capable of mineralising 4-aminobenzene-sulfonate (4ABS). *Hydrogenophaga palleronii* S1 was able to oxidise 4ABS. However, growth was poor on this substrate and the metabolite accumulated. *Agrobacterium radiobacter* S2 was capable of utilizing only oxidised 4ABS. Together, the growth of both organisms were enhanced. By the addition of 4-aminobenzoate, biotin and vitamin B12, S1 was capable of growth on 4ABS alone. The authors suggested that S2 provides these nutrients for S1 which allows S1 to grow and oxidise 4ABS to be used by both organisms.

Commensalistic relationships (0 +) occur when one organism gains benefit from the association while the other is not affected. This relationship seems to be the most common association found in interactions in the soil community mainly because it is easier to confirm. Brodkorb and Legge (1992) found that in one soil system the addition of the white rot fungus, *Phanerochaete chrysosporium*, enhanced the mineralization of phenanthrene in the soil. They suggested the following reason for this increase in mineralization. The peroxidase released by the fungus hydroxylates the PAH making it more polar. The increase in polarity would increase the solubility and, thus, bioavailability of the PAH thereby making the PAH more readily metabolised by the native community.

A commensalistic relationship was also found in the mineralization of cyclohexane (Beam and Perry, 1974). *Mycobacterium vaccae* JOB5 was capable of cometabolizing cyclohexane to cyclohexanone while growing on propane as a cabon and energy source. The other bacterium in this coculture, strain CY6, was capable of mineralizing cyclohexanone but not cyclohexane nor propane.

Likewise, cometabolic transformations of large PAHs are postulated as the basis for commensalistic relationships but few mixed cultures confirming this appear in the literature. Much research shows large PAHs to be cometabolized by a single culture but few isolates growing on the cometabolized substrate have been confirmed.

Amensalism (0 -) occurs when one species retards the other while the former is unaffected. These interactions may occur during the release of antibiotics into the soil to retard the growth of sensitive cells. Unlike Brodkorb and Legge (1992), Radtke et al. (1994) detected factors in the soil that suppress remediation when *P. chrysosporium* is added to the soil. They found that under high nitrogen and neutral pH conditions the growth of the white rot fungus was strongly inhibited. Under these conditions *Pseudomonas* sp. in the soil produced phenazine-1-carboxylic acid which is a fungistatic antibiotic. However, under acidic conditions and low nitrogen levels in the soil, this

inhibition was not as debilitating to the growth of the fungus. Indeed, environmental soil conditions can exhibit an important role in community interactions.

When two organisms are inhibiting each other, their interaction is competitive (- -) and neither organism benefits from this relationship. This interaction occurs when both organisms are competing for the same carbon and energy source. Generally, competition increases as the similarity between the two organisms increases (Fredrickson and Stephanopoulos, 1981). Interest in this particular interaction has been extensive since this is the basic mechanism governing natural selection (Gause, 1934). When two organisms are growing on the same substrate, the one with the highest growth rate has a competitive advantage (Slater, 1979). This interaction, though easy to confirm in the laboratory, is difficult to confirm in natural communities where multiple substrates exist and interactions with other organisms in the community may take precedence over the competition between these two species.

Parasitism/predation (+ -) occurs when one species gains benefit from the other by using it as host or prey. Parasitism refers to a relationship where the parasite is smaller than the host and predation refers to the interaction when the predator is larger than the prey. Predation often occurs in the soil community with protozoans as the predator of bacteria (Janzen et al., 1995). As would be expected, overall removal of contaminants from the soil by bacteria would be decreased in this relationship.

Neutralism is very difficult to evaluate and is poorly researched. Some explanations for neutralistic interactions may be that the populations are physically separated or the requirements for growth of each organism are so different that neither population alters the requirements of the other (Bull and Slater, 1982).

1.6 Bioremediation of PAH-contaminated soil

Bioremediation is defined as the treatment of contaminated sites by enhancing the growth of microorganisms capable of degrading these contaminants to harmless products.

As shown previously, microorganisms can mineralize PAHs and other components of creosote in optimal, controlled conditions. But, in the soil environment many factors decrease this rate of degradation.

Laboratory studies are necessary for determining if the environment contains organisms capable of removing the contaminant, if non-native microorganisms may be necessary to facilitate removal and if the products of this metabolism are less toxic than the parent material (Gieg, 1996). Other considerations to be taken into account before bioremediation is deemed appropriate are 1) the site must not contain chemicals or combinations thereof that are inhibitory to degradation 2) the target chemicals must be available to the population 3) conditions must be amenable to microbial growth and activity and 4) the cost of bioremediation must not be more than other remediation technologies (Alexander, 1994). Overall, bioremediation is beneficial because contaminants are completely mineralized to CO_2 and water, bioremediation is inherently versatile and can be coupled to other treatments, eliminates long term liability and is cost effective (Cutright and Lee, 1995).

Although many physical and chemical means of contaminant removal exist (Table 1.1), bioremediation is usually selected as the final "polishing touch" to clean up sites. Chemical and physical methods are most efficient when used to clean up heavily contaminated sites while bioremediation is applied to sites typically containing less than 2% contamination. At this low level, physico-chemical techniques would be less cost effective than bioremediation. Above this level of contamination, organisms used to remediate the soil are inhibited by toxic effects of the contamination such as pentachlorophenol, heavy metals or heterocyclic compounds which often accompany PAH pollution. Old hazardous waste sites usually have decreased toxicity due to volatilization or humification of some of the toxic components.

Because of the greater understanding of factors affecting organisms in soil, better methods for removal of PAHs have been devised. It has been estimated that the cost of

classic remediation techniques, such as incineration, would be high: about $20 million for remediation of a typical Superfund site in the U.S.A. Biological treatment of such a site is estimated to decrease this cost by approximately ten-fold (Fiorenza et al., 1991).

A number of remediation techniques have been developed for the treatment of hazardous wastes (Table 1.1). Each of these techniques has a selective advantage over the others depending on the type of contamination and soil environment. Due to the cost effectiveness of certain remediation methods under various conditions and the complexity

Table **1.1** Physical, chemical and biological treatment technologies for contaminated soils (adapted from Bull, 1992)

Physical	***Biological***
Soil washing	Landfarming
Air (Steam) stripping	Prepared bed reactors
Activated charcoal treatment	Composting/humification
Ion exchange	Bioenrichment
Vitrification	Bioaugmentation
Incineration	Slurry Bioreactors
Chemical	Anaerobic or biofilm reactors
Electrokinetic removal	Bioslurping
Solvent extraction	Bioventing
Ozonolysis	Biosparging

of contamination in the soil, the most effective process for cleanup would involve linking physico-chemical processes to biological treatments. Whatever the treatment used, four steps should be followed to determine which strategy would be most adequate : 1) site evaluation in order to understand the extent of contamination and problems that may be encountered, 2) assessments to determine treatability of contaminated sites and optimization

of the process, 3) full scale treatment design and 4) a way of monitoring the treatment process (Ellis et al., 1991).

Although bioremediation has gained much attention in the area of soil cleanup only recently, it has been used in the remediation of oily sludges by petroleum industries for 30 years (Ryan et al., 1991). Landfarming involves the spreading of contaminated sludges on the land as a slurry and allowing the indigenous populations to mineralize or humify the wastes. The rates of remediation are enhanced by the application of nutrients, occasional tilling to improve aeration, irrigation and liming to maintain optimal soil pH. This means of remediation is neither labor nor energy intensive and therefore provides the lowest cost of remediation. This, and other forms of biological treatment can be classified into 3 general groups: solid phase bioremediation, *in situ* bioremediation and slurry bioreactors.

1.6.1 Solid phase bioremediation

Solid phase bioremediation techniques are useful when treating soils containing sorbed chemicals such as PAHs (Fiorenza et al., 1991). Landfarming is the simplest form of solid phase bioremediation. The major disadvantage of landfarming is the lack of containment of the waste and its more soluble metabolites (Wilson and Jones, 1993). Therefore, recently, there has been a shift towards the development and use of prepared bed reactors.

In this system, soil is excavated and a liner of low permeability material is applied around the resulting pit to prevent mobility of the contaminant. Sand is usually placed on top of the liner to improve drainage and a system to collect the leachate is also installed (Alexander, 1994). Contaminated soil is excavated and amended with water, nutrients, and lime and returned to the lined treatment facility. Often, a perforated piping system spans the unit to provide adequate air to facilitate rapid removal of the contaminant. These units are usually covered to prevent emissions of volatile wastes and to control the temperature and moisture. The leachate is collected and treated by another biological means such as

trickling bed reactor or chemically treated by passing the leachate through an activated charcoal filter and the clean liquid can then be recycled.

The overhead cost of excavating and enclosing an area in a liner may initially increase the cost of the process. However, detoxification rates are usually enhanced compared to landfarming and the possibility of contamination moving off-site is reduced making this system a widely used treatment technology today. There are extensive reports of the utilization of land treatment technologies for the removal of creosote contaminated sites (Devine, 1995)

Another solid phase method is composting bed reactors where remediation at higher temperatures sometimes allows for enhanced removal of certain compounds (Alexander, 1994). The contaminated sludge is mixed with bulking agents such as straw or wood chips and is aerated by either forced aeration or pile turning. Composting is usually used for highly contaminated sludges but rarely with hydrocarbon contaminated soils (Wilson and Jones, 1993). Kastner et al., (1995) have found that addition of compost to highly contaminated soils also increases the rate of humification, and therefore detoxication, of PAHs and other wastes.

1.6.2 *In situ* bioremediation

The purpose of *in situ* bioremediation is to stimulate hydrocarbon degrading bacteria in the vadose and saturated zones of the soil (Pollard et al., 1994). The contaminated soil is essentially undisturbed and therefore the fear of spreading contaminated soil by excavation and transport are minimized. Usually a series of wells are drilled throughout the contaminated area and amendments are added through these wells to enhance indigenous or allochthonous microorganisms. Down gradient from this site, groundwater is recovered and recirculated after some form of surface treatment. In this way, off site contamination of mobile wastes are prevented (Pollard et al., 1994). Wells

are often set up between the addition sites and the recovery sites to determine the extent of remediation, cell numbers and nutrient levels.

However, *in situ* techniques are dependent upon environmental factors. These treatments are also very dependent upon the permeability of the soil and the uniformity of the soil to permeation by nutrients and oxygen. For example, if contamination occurs within a patch of the subsurface with a higher proportion of clay than the surrounding area, then water and amendments will bypass the contaminated area. Generally, sites with permeabilities less than 10^{-6} m/s should not apply this technology (Thomas et al., 1987).

Oxygen is the primary limiting factor to *in situ* bioremediation. Oxygen is present at low concentrations in groundwater and has low solubility in water. Therefore addition of air down the wells would be the largest single cost to *in situ* treatments. To reduce this cost hydrogen peroxide is often added to the soil because it slowly degrades to water and oxygen and is highly soluble in water (McCarty, 1988). Concentrations greater than 100 - 200 mg/L may be toxic to microorganisms and caution must be taken when adding H_2O_2 to soil. Nitrate, because of its low cost, can also be added to the soil as an alternate electron acceptor for PAH degradation (Mihelcic and Luthy, 1988a, 1988b) but care must be taken for nitrate becomes a pollutant itself when present in drinking water above 10 mg/L (Alexander, 1994).

In situ techniques are not usually chosen as a method for remediating PAH contaminated sites due to their insolubility and adsorptive properties. However, Ellis et al., (1991) chose this technique for remediating a creosote contaminated site for several reasons. The site was located beside a lake and excavation may have caused contamination of the lake and the site was currently being developed as a residential neighborhood. After a year - long feasability study, the *in situ* treatment was applied. Groundwater retrieved down gradient from the site was treated in a biological reactor inoculated with PAH degraders and recycled through the system. Most of the small PAHs were removed; however, large PAHs tended to persist.

1.6.3 Slurry bioreactors

In this treatment system contaminated soil is excavated and added in batch mode, as an aqueous slurry, into a specific reactor. There is a high degree of process control and this allows for reduced treatment times relative to other forms of bioremediation. Often, an acclimatized population may be added from a previous batch to further accelerate biodegradation rates.

Slurry formation of the soil and adequate mixing enhance the availability of PAHs to the population by breaking clay aggregates and SOM and exposing the otherwise unreachable contaminant to the microorganisms. Because of the limitations of both in situ and solid phase treatment technologies, slurry bioreactors may have applications in the treatment of high clay content soil (Pollard et al., 1994). This method is also gaining more attention because it may be able to overcome the difficulty of removing large PAHs from the environment if the appropriate mixed culture is acclimatized (Mueller et al., 1993).

The proportion of soil that can be treated in the slurry per batch depends upon the soil composition and the type of aeration of the bioreactor. One form of slurry reactor is the airlift bioreactor which uses air as a mixing agent by pushing air from the bottom of the reactor to the top. In sand soils, particles tend to settle quickly after being disrupted and mixing can be poor. The difficulty with this technology is that many air lift bioreactors can not exceed 20% soil content in the slurries. Above this limit, mixing and aeration of the slurry becomes inefficient and energetically costly. This increases costs to the treatment by using high proportions of water and low proportions of contaminated soil. Sometimes the water can be recycled but the low soil content in the slurry increases the time of overall site remediation.

Recently, Gray et al. (1994) utilized a rotating drum bioreactor which consisted of a high solids (60%) content slurry with an acclimatized anthracene degrading culture. This design was capable of removing 200 µg anthracene per gram of soil per day. Rotating

drum bioreactors also reduce the cost of treatment by expending less energy for adequate aeration and mixing within the slurry.

1.7 This research

The research presented in this thesis focussed on the interactions of bacteria with each other and the soil matrix found in the roller drum bioreactor developed by Gray et al. (1994). From this previous research, a community of bacteria was enriched from a creosote contaminated soil which was capable of removing anthracene at a rate of 200 $mg \cdot kg\ soil^{-1} \cdot day^{-1}$ using a 60% soil slurry in a 2 L roller bottle. Similar results were obtained when the process was scaled up to a 14 L rotating drum bioreactor (Bannerjee et al., 1995). In addition, the established community was able to degrade 95% of the anthracene over 18 transfers to anthracene contaminated soil. From this active population, two distinct sub-populations were identified on complex agar: a fast growing, Gram negative pseudomonad-like population and a slow growing, Gram positive, acid-fast population characteristic of actinomycetes. Other research has also found that the actinomycetes may play an extremely important role in PAH degradation in contaminated soils (Kastner et al., 1994).

All colonies isolated from each subpopulation had very similar colonial morphologies. It appeared that this mixed culture was composed of one fast growing and one slow growing isolate, based solely on colonial morphologies. The slow growing population outnumbered the fast growing population by 100 fold when anthracene served as the carbon source. Based on the growth rate of each organism, one might expect that the fast growing population would outnumber the slow growing population if these organisms were competing for the same initial substrate, anthracene.

From the slow growing population, one isolate was characterized as *Rhodococcus* S1 (Tongpim and Pickard, 1996). This isolate was capable of growth only on anthracene and its 2-chloro- and 2-methyl- derivatives suggesting a very narrow substrate specificity.

Other slow growing isolates from this culture had very similar physiological and biochemical properties to *Rhodococcus* S1. From the fast growing population a soil pseudomonad was isolated, but it was not able to grow as a pure culture on anthracene. This suggested that the interaction between these two populations may be either cometabolic or the pseudomonad may be nutritionally dependent upon the slow growing population.

It was previously shown that a pure culture of *Rhodococcus* S1 grew very slowly on anthracene in liquid culture (Tongpim and Pickard, 1996). The objective of this research was to determine which factors enhanced the rate degradation of anthracene observed in the original mixed culture when compared to a pure culture of *Rhodococcus* S1. It was suspected that either the dissolution rate of anthracene or the interactions of *Rhodococcus* S1 with other members of the enriched population enhanced the rate of degradation of anthracene to that which was observed by Gray et al. (1994).

To determine the effect of either of these factors several experiments need to be performed: (i) the soil system in the roller drum bioreactor must be simplified to minimize other interactions between the soil, bacteria and anthracene, (ii) the dissolution rate of anthracene in the absence of degrading bacteria in this model soil will be compared to the dissolution rate in liquid culture, (iii) the interactions of S1 with both the anthracene and soil will be monitored, (iv) the anthracene removal rates by *Rhodococcus* S1 in the roller drum bioreactor will be compared to that found in liquid culture; (v) a fast growing *Pseudomonas* sp. will be added to a pure culture of *Rhodococcus* S1 and the relationship between these two organisms in a roller drum bioreactor will be determined by monitoring the removal rates of anthracene and growth of each isolate and finally (vi) since it is suspected that the *Pseudomonas* isolate may use a cometabolite produced by the original mixed culture, metabolites from a mutant *Pseudomonas* strain will be extracted and provided as a carbon source to the pseudomonad both in the presence and absence of other

substrates. The methods and results of these studies are presented in the following chapters.

2. MATERIALS AND METHODS

2.1 Chemicals and materials used

All fine chemicals including PAHs, naphthoic acid and hexadecane used (usually >98% purity) were obtained from Sigma Chemicals (St. Louis, USA). Dichloromethane for extractions was purchased from EM Science (Gibbstown, USA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Nepean, Canada).

Glass beads were purchased from Sil Silica (Edmonton, Canada) and 0.22 µm membrane filters were obtained from Millipore. All container parts that might come in contact with dichloromethane were made of either glass or teflon. Before starting experiments, all glassware was rinsed with dichloromethane.

2.2 Preparation of media

Trypticase soy broth was obtained from Becton Dickinson (Cockeysville, USA) while Plate Count Agar and Noble Agar were from Difco (Detroit, USA). API 20E strips were purchased from Biomerieux (Charbonnières des Bains, France).

The minimal salts medium (B+N8P) contained (per 900 mL distilled water): 1.0 g NH_4Cl, 2.0 g KNO_3, 2.0 g Na_2SO_4 and 0.05 g $FeSO_4.7H_2O$. A solution of 40 g/L KH_2PO_4-K_2HPO_4 buffer at pH 7.2 was prepared by combining 300 mL of 40 g/L KH_2PO_4 with 1 L of 40 g/L K_2HPO_4. This phosphate buffer (100 mL) was added to the minimal salts medium along with 1 mL of a trace metal solution containing the following components (g/L): $CaCl_2 \cdot 2H_2O$, 3.7; H_3BO_3, 2.5; $MnCl_2$, 0.87; $FeCl_3$, 0.65; $ZnCl_2$, 0.44; $Na_2MoO_4 \cdot 2H_2O$, 0.29; $CoCl_2$, 0.01 and $CuCl_2$ at 0.1 mg/L. The B+N8P medium (200 mL) was dispensed into 500 mL Erlenmeyer flasks containing 25 cm stainless steel coil with 1 cm diameter around the bottom to break up clumps of cells and anthracene. Ground anthracene (100 mg) was added and the flask was closed with a cotton plug. The medium was sterilized by autoclaving for 20 min (121°C) and, after cooling, 2

mL of sterile 0.1 g/mL $MgSO_4 \cdot 7H_2O$ was added. To prepare minimal medium agar, 1.5% noble agar was added to the medium before autoclaving.

Milloning's buffer containing the following in 1L of water (g): $NaH_2PO_4 \cdot H_20$, 16.8; NaOH, 3.86; glucose, 5.4; $CaCl_2$, 0.05 (Sanders et al., 1975) was used to wash glutaraldehyde from the samples prepared for Scanning Electron Microscopy (SEM).

2.3 Isolation of pure cultures

Rhodococcus S1, isolated and characterized as described by Tongpim and Pickard (1996) and Gray et al. (1994), was supplied by S. Tongpim. The gram negative isolate was isolated from the same creosote contaminated soil by A. Hashimoto. Characterization of the gram negative isolate was determined using the multitest API 20e strip.

Gram positive isolate A1 was isolated from an enrichment culture of creosote degrading microbes. This mixed culture was able to utilize anthracene as sole carbon and energy source. After three weeks, the mixed culture was spread onto solidified minimal salts agar. One day after inoculation on minimal salts agar, a 1% solution of anthracene in dichloromethane was sprayed onto the surface of the plate as a visible uniform layer. Colonies capable of degrading anthracene were identified by a clearing halo surrounding the colony after 2 weeks (Bogardt and Hemmingsen, 1992). These isolated colonies were confirmed to be pure by transferring three times to either minimal salts medium agar or PCA and sprayed with anthracene. A pure culture was transferred to 200 mL minimal salts medium to develop maintenance and stock cultures.

The ability of Gram positive isolate A1 to utilize other PAHs was also tested. One day after inoculation, the compounds were uniformly sprayed onto the solid medium. Utilization of the PAH was followed by observing growth of colonies and zones of clearing surrounding these colonies. The plates were compared to an inoculated control plate not sprayed with any PAHs to confirm that the isolate was not using agar as a carbon and energy source.

2.3.1 Storage and maintenance of cultures

For long term storage, cultures capable of metabolizing anthracene were grown in anthracene salts medium, centrifuged and rinsed in phosphate buffer. Centrifugation and rinsing was repeated two times. The resultant pellet was diluted in 20% glycerol to approximately 10^9 cfu/mL and frozen in 1.0 mL samples at -80°C.

For a routine maintenance culture, 0.1 mL of the frozen stock was inoculated into 200 mL of basal medium with anthracene as the only carbon and energy source. After 2 weeks, the culture was streaked on PCA and grown at 27°C for 2 or 7 days before observing colonies for purity. If the culture was pure, it was used for monthly transfers.

To prepare inocula for biodegradation studies, 0.1 mL of glycerol - stored cultures were inoculated into 200 mL minimal salts medium supplemented with 0.05% (w/w) crushed anthracene in 500 mL shake flasks containing the stainless steel coil. The flasks were incubated in the dark at 30°C on an orbital shaker at 200 rpm for two weeks. This culture (10 mL) was added to 140 g of glass beads coated with anthracene and 70 mL minimal salts medium. Initial cell numbers were counted either by determination of colony forming units by serial dilutions on PCA or by direct measurement (Petroff Hauser counting chamber).

2.4 Preparation of glass beads

The substitute soil system used in all the experiments were glass beads #3 obtained from Sil Silica Inc. The glass beads were separated into uniform size ranges. Glass beads with 500 - 1000 µm diameter were separated using 2 soil sieves courtesy of the Department of Renewable Resources at the University of Alberta. The first sieve allowed particles less than 1000 µm (60 mesh) to pass through. Underneath, the second sieve removed particles less than 500 µm (30 mesh). One kilogram of glass beads was added to a 1000 µm sieve and the sieves were shaken on a rotary shaker at 280 rpm for 15 min. Then 800 g batches of glass beads were acid washed in 750 mL of 5-7% HNO_3 at 27°C overnight to remove

the alkaline residues used to prepare the glass beads. The glass beads were allowed to settle and the murky supernatant was decanted. The remaining supernatant was vacuum filtered through a sintered glass filter and the glass beads were rinsed with 1.5 L of distilled water four times. To bring the pH to neutrality, 20 mL of phosphate buffer (pH 7.2) was passed through the glass beads followed by a final rinse with distilled water. The water was removed by vacuum filtration of the glass beads for 2 h and the glass beads were allowed to air dry overnight (or longer). To remove organic matter from the surface of the glass beads and to sterilize the glass beads, 1 kg batches of air dried beads were wrapped in aluminum foil and heated to 550°C in a muffle furnace for 24 h.

2.4.1 Coating glass beads with anthracene

Anthracene was dissolved in dichloromethane at a concentration of 10 mg/mL and 30 mL was added to a sterile 2-L roller bottle (mason jar) containing 420 g sterile glass beads. To allow adequate air exchange, air holes were placed in the aluminum sealer of the roller bottle. This was covered with filter paper to maintain sterility. The roller bottle was rolled for 2 days at 3 rpm on a Bellco Biotechnology Cell-production Roller Apparatus to ensure adequate coating of the glass beads with anthracene and to allow for complete evaporation of the solvent.

Because most PAHs fluoresce, the glass beads were observed under long wave U.V. light to confirm that they were coated with anthracene. The efficiency of coating the glass beads was determined by extraction and quantification of the anthracene from the glass beads and from the roller bottle, carried out separately.

2.5 Experimental conditions

The concentration of anthracene desired in the glass bead slurry was 100 µg/ 200 g slurry based on the solid:liquid ratio required to adequately tumble in the roller bottle.

Therefore, 70 mL of minimal salts medium was mixed with 140 g anthracene coated glass beads. For *Rhodococcus* S1 experiments, three week old maintenance cultures were used. Ten mL of the three week old culture (approximately 10^7 cfu/mL) was added to the glass bead slurry. To allow adequate mixing the slurry was rolled for 1 h before samples were assessed for viable counts and for initial anthracene concentrations. Initially, samples for viable counts and anthracene concentrations were taken on days 0, 1, 2, 4, 6, and 7. After day 7, the experiment was either terminated or samples were taken once weekly thereafter.

Because *Rhodococcus* S1 has a tendency to attach to the glass surface and to clump, determination of cell biomass by measuring the absorbance of the culture at 600 nm proved to be poorly reproducible under these conditions. Therefore, one gram of slurry was taken for viable counts and added to 9 mL of 0.1% peptone solution. After 1 h in 0.1% peptone, the sample was vortexed and serially diluted to 10^{-6}. Samples (0.1 mL) were plated onto plate count agar in triplicate and incubated at 27°C for 7 days for determining *Rhodococcus* S1 numbers or 2 days for ascertaining gram negative cell numbers.

To follow the reduction of anthracene concentrations, 15 g of slurry was aseptically removed from the roller bottle. The sample was vacuum filtered through a 0.45 µm filter to remove the minimal salts medium but retain any crystals sloughed off the glass beads during incubation. After 5 min filtration, the glass beads and filter were weighed and further dried by the addition of 15 g anhydrous Na_2SO_4. The dried glass beads were added to a cellulose extraction thimble (Whatman, Maidstone, England). The thimble was extracted with 200 mL dichloromethane in a Soxhlet apparatus for four hours. The resulting extract was concentrated to 5 mL by rotary evaporation at 60°C. For GC analysis, 1 mL of concentrate was added to 1 mL of internal standard (hexadecane) and 2µL was injected onto the column. For HPLC, the sample was evaporated and redissolved in acetonitrile. Internal standard (naphthoic acid, 40 µg) was added to 20 - 60 µL of concentrate made up to 100 µL with acetonitrile and 10 µL was injected.

2.6 Scanning Electron Microscopy

Glass beads for Scanning Electron Microscopy (SEM) were prepared and coated with anthracene as described earlier. *Rhodococcus* S1 was grown in the presence of glass beads and anthracene. Two grams of sample (moist glass beads and anthracene) were removed from the roller bottle. The liquid was removed and the beads and anthracene were fixed with 2.5% (v/v) glutaraldehyde in Milloning's buffer for 18 h at 5°C. The glutaraldehyde solution was then removed by pasteur pipet and the glass beads were washed with fresh Milloning's buffer twice. The samples were almost completely dehydrated using pure ethanol in distilled water solutions for 15 min intervals using 30%, 50%, 70%, 90% and 90% ethanol/water mixtures. Preliminary experiments had shown that dehydration with 100% ethanol removed all anthracene from the glass beads. The beads were air dried for 48 hours. Carbon coated doubled-sided tape was placed onto a metal stud and the glass beads were placed on the tape as a single layer. The SEM studs were sputter coated with gold using a Hummer-I™ sputter coating apparatus. The samples were viewed under a Field Emission JEOL 6301FXV Scanning Electron Microscope at an accelerating voltage of 2.5-10 kV. The electron microscopy was performed by Mr. G. Braybrook, Department of Earth and Atmospheric Sciences at the University of Alberta.

2.7 Radioactive experiments

The methodology of the radioactive experiments conducted in this thesis were similar to those performed by Stucki and Alexander (1987). The specific activity of [9-^{14}C]-anthracene was 15.1 mCi/mmol. In these experiments, the roller bottle assays were scaled down to 18 x 150 mm roller tubes containing 17.5 g glass beads and 10 mL of minimal salts medium. Each test sample was prepared in triplicate. Radioactive anthracene (30 µL; 267480 dpm; 1.50 µg) in benzene was added to the glass beads in the roller tube. The tubes were rolled at 3.5 rpm at room temperature for two hours to allow the benzene to

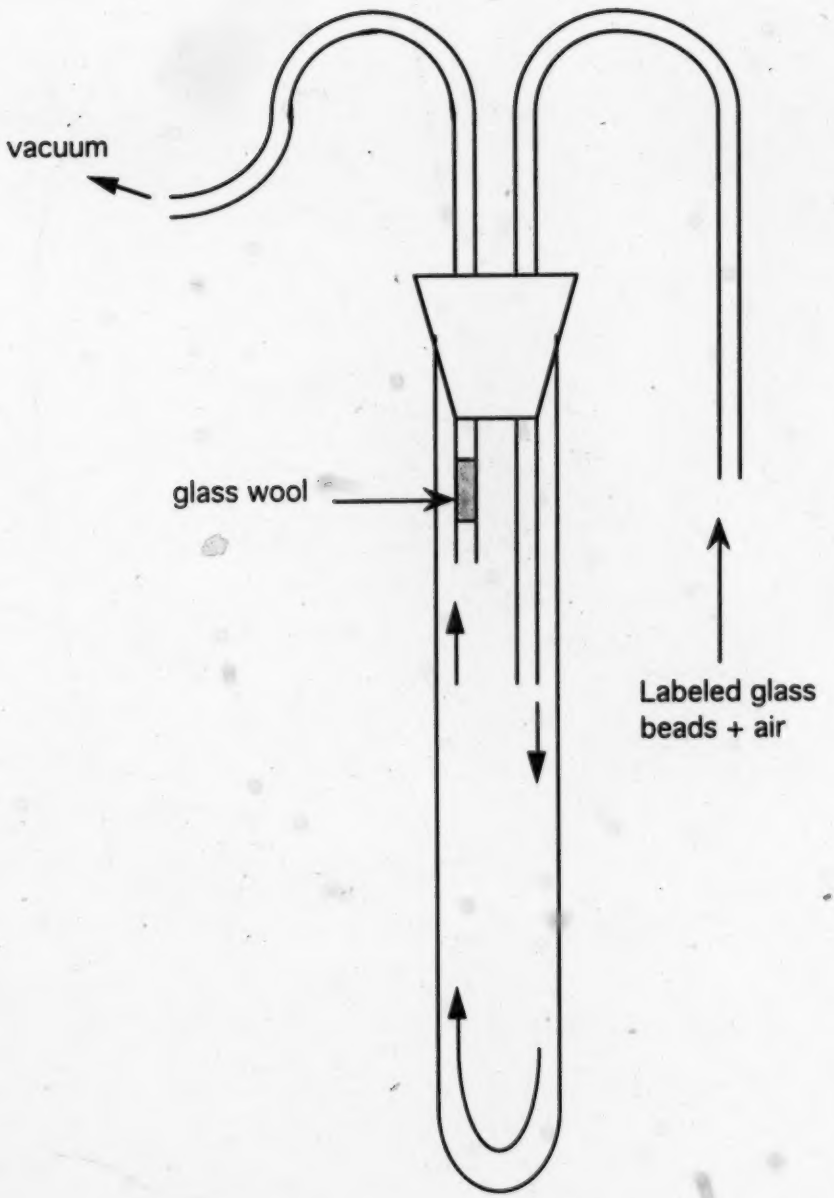
evaporate. Then, 10 mL of minimal salts medium was added to all the samples. The samples were rolled at 3.5 rpm for 1 h and 1 mL aliquots were taken as initial samples. The sample was filtered through a 0.2 µm filter to remove any particulate anthracene and allow passage of only soluble anthracene. The filtrate (1mL) was added to 10 mL of ACS fluid and counted by liquid scintillation. Triplicate samples were taken at various times over four or seven days.

To test the effect of surface area alone on the solubility of anthracene, the test was slightly modified. In some tubes, glass beads (1.9 g) were added which were calculated to have the same approximate surface area as the inner surface of the roller tube which came in contact with the medium. In this experiment, 30 µL or 100 µL of [9-^{14}C]-anthracene in benzene was added to 470 µL or 400 µL of dichloromethane, respectively. This was added to either 1.90 g of glass beads (just enough to moisten the beads) or to a 18 x 150 mm test tube. The test tubes were rolled and the glass beads were mixed until the dichloromethane had evaporated. To prevent the loss of any glass beads and radioactivity when transferring to the roller tube, a vacuum apparatus like that in Figure 2.1 was used. Briefly, two hollow glass rods with an internal diameter of 5 mm were inserted into a 150 mm (#2) rubber stopper. The suction port was stopped with glass wool to prevent radioactive glass beads from entering the vacuum system. This apparatus was placed onto the appropriate roller tube and the vacuum was applied until all the glass beads were in the test tube. Ten mL of B+N8P was added to all samples and 0.5 mL aliquots were taken and counted as mentioned above at appropriate time points.

2.8 Preparation of phenanthrene dihydrodiol

Pseudomonas fluorescens strain LP6a mutant D1, known to degrade phenanthrene to its corresponding dihydrodiol, was obtained from Dr. J. Foght in this department. In this mutant, the enzyme required to further metabolize the phenanthrene dihydrodiol had been interrupted by a kanamycin resistance gene and kanamycin resistance was used for

Figure 2.1 Vacuum apparatus used to transfer radioactive glass beads to roller tubes. The arrows indicate the direction of air flow when a vacuum was applied to the system.


vacuum
glass wool
Labeled glass
beads + air

selection (Foght and Westlake, 1996). This culture was maintained on Trypticase Soy agar with kanamycin (50 µg/mL) by incubation at 30°C for 2 days and stored at 4°C with monthly subculturing.

To produce the phenanthrene dihydrodiol, this organism was grown in a 500 mL shake flask with 200 mL of trypticase soy broth (TSB) containing 50 µg/mL kanamycin. The culture was incubated at 27°C overnight, shaken on a rotary shaker at 250 rpm. Twenty-five mL of the culture was transferred to each of 6 - 2 L flasks, containing 1 L TSB with 50 µg/mL kanamycin and shaken for 1 day. To induce the cells for production of their dioxygenase, 0.5 µmol salicylate was added to each flask and the culture was shaken for 3 hours. The cells were harvested by centrifugation at 8000 rpm and resuspended in 3 mM phosphate buffer (pH 7.2). Aliquots were added to 6 flasks containing 1 L of 2.5 mM phenanthrene in 3 mM phosphate buffer and shaken for 1-3 days. The cells were pelleted by centrifugation and the supernatant was further cleared by membrane filtration using a 0.22 µm Millipore filter. The resulting supernatant was extracted 3 times using 250 mL dichloromethane/ L supernatant. Approximately 1.25 g of dihydrodiol were produced from 2.7 g phenanthrene. The purity of the extract was analyzed by GC, HPLC, and thin layer chromatography (TLC).

Coating the glass beads with the phenanthrene dihydrodiol was carried out by a very similar procedure for labeling glass beads with anthracene. Because of the relatively low quantities of dihydrodiol, the experiments were scaled down. In these experiments, 10 g of glass beads, labeled with 5 mg of phenanthrene dihydrodiol, were added to 18 X 150 mm test tubes along with 10 mL of minimal salts medium. Test tubes were placed on a rolling apparatus rotating at 3.5 rpm. The apparatus was covered with aluminum foil to prevent photooxidation of the dihydrodiol. For a sample, the entire contents of one test tube were extracted with dichloromethane in a separatory flask and passed through anhydrous sodium sulphate. Each sample was done in triplicate. In all these studies, abiotic controls were compared to inoculated samples. All samples were analyzed by

HPLC. Removal of the dihydrodiol was monitored by extracting samples at day 0, and after 14 and 21 days.

2.9 Analytical Procedures

All analyses followed the loss of anthracene or dihydrodiol over time by either GC or HPLC. For GC, 1 mL of internal standard (hexadecane, 2g/L) was added to 1 mL of the concentrated sample. If expected levels of anthracene in the concentrate were too high, the sample was diluted to approximately 1 mg/mL. The sample was analyzed by a Hewlett Packard 5730A capillary gas chromatograph connected to a Hewlett Packard 3390A Integrator. Helium (80 psi) was used as the carrier gas and anthracene was detected by a flame ionization detector (FID). The injected sample (2 µL) was split 20:1 and passed through a DB-5 30 m X 0.25 mm (internal diameter) column (0.25 micron film, J&W Scientific, California) with the following oven program: 90°C for 2 minutes then 16°C/min to 250°C and 4 min at 250°C. For analysis of the phenanthrene dihydrodiol, the following program was used: 90°C for 2 min; 2°C/min to 250°C; 250°C for 2 min.

The solvent system used to separate the phenanthrene dihydrodiol during thin layer chromatography consisted of benzene/acetone/acetic acid (85/15/5). Aliquots (1 µL, 5 µL or 10 µL) were spotted onto a 20 X 5 cm sheet of silica gel with fluorescent indicator (Kodak #13181). After the solvent reached 1 cm from the top, the sheet was removed from the developing chamber, dried and examined under U.V. light.

Most analyses which involved the phenanthrene dihydrodiol were monitored by HPLC. When monitoring the phenanthrene dihydrodiol, anthracene was used as the internal standard and when quantifying anthracene levels, naphthoic acid was the internal standard. If expected levels of anthracene or phenanthrene dihydrodiol in the concentrate were above 0.2 µg/mL, the sample was diluted with acetonitrile. The sample was mixed with internal standard as previously described and analyzed by a Waters HPLC pump

equipped with a Waters 712 WISP autosampler and a Waters 486 tunable absorbance detector connected to a Hewlett Packard 3392A Integrator. Separation of phenanthrene dihydrodiol occurred with a mobile phase consisting of acetonitrile/water/acetic acid ratio of 45/55/1 via isocratic elution (Wilkinson et al., 1996). The mobile phase was filtered through 0.45 µm solvent tolerable filter (Whatman Type HA) to remove particulate matter. When monitoring anthracene levels only, the mobile phase was changed to 70/30 (acetonitrile/water). Quantification of the PAH was performed by UV detection at 254 nm after separation on a Brownlee Spheri-10 RP-18 reverse phase column (100 X 4.6 mm; Brownlee Laboratories, Calgary, Canada).

3. RESULTS

The fundamental goal of this research was to understand interactions within a reactor during the bioremediation of PAH contaminated soil, specifically, during the degradation of anthracene in the roller drum bioreactor. In the original experiments by Gray et al. (1994), anthracene was coated onto soil and an enriched culture capable of degrading anthracene was established. This mixed culture was capable of removing 600 mg anthracene/kg soil in three days. As described previously, this mixed culture consisted of two predominant colonial morphologies on complex agar: a fast growing, Gram negative population seen after two days growth and a slow growing Gram positive, acid-fast population observed after 7 days growth. A pure culture of a slow growing isolate, *Rhodococcus* S1, removed anthracene at a much lower rate than the mixed culture when grown in liquid culture exhibiting a doubling time of 24 h (Tongpim and Pickard, 1996). Thus, one objective of this research was to determine the discrepancy between the ability of the pure culture and the mixed culture to degrade anthracene.

3.1 Selection and characterization of the soil system

In order to investigate a reason for this discrepancy between pure cultures and mixed cultures, a suitable soil system was required. A suitable soil was chosen by monitoring the physical interactions of anthracene with the selected soil in order to establish the behaviour of anthracene with the soil. An important preliminary step required before the interactions of bacteria in an anthracene contaminated soil system were followed was to first understand the interactions of the substrate with the soil. Many interactions of PAHs with clay and organic matter are complex and difficult to follow. The complexity of these interactions are primarily due to the large surface area of clay while the hydrophobic nature of PAHs further complicates the interactions by causing adsorbtion of PAHs to SOM.

3.1.1 Assessment of sand as the model soil.

In this project, sand was initially chosen as the surrogate soil because it does not aggregate, contains only low levels of SOM, has a relatively homogeneous particle size and it does not irreversibly sorb PAHs. It also permits adequate oxygen exchange due to its large size and, assuming spherical particles, the approximate surface area is readily calculated due to the absence of pores within the particles.

Sand was obtained from a landscaping retailer in Edmonton, Canada. After sieving the sand to a size range of 0.25-1 mm and washing with distilled water, it was added to minimal salts medium (B+N8P) at different ratios to determine the optimal conditions which gave the best mixing as observed visually in a roller bottle. Optimal conditions were defined as a minimum of salts medium that allowed the sand to tumble in the roller bottle rather than moving as a single mass. It was found that a ratio of 140 g sand: 80 g B+N8P gave optimal mixing conditions. However, during experimentation, gradual leaching of minerals from the sand into the medium caused the pH to rise to 9.3. The sand was also not characterized as to the types of metals and minerals that were present which may further affect bacterial interactions in this soil ecosystem.

Although highly purified sand was available, it was decided that the potential for similar problems could be avoided by using a single, relatively pure component of sand, silica. Thus, the soil component of the slurry in the roller bottle experiments was changed to glass beads. The glass beads were sieved to a size range of 0.425 mm - 1.0 mm and acid washed to remove organic matter and minerals. After rinsing, they were air dried and sterilized in a muffle furnace at 550°C.

3.1.2 Preparation of glass beads

When determining the surface area of the glass beads, the density was assumed to be equivalent to an average sand particle (2.65 g/cm^3) and the shape was assumed to be spherical. From this, the total surface area of 1 gram of glass beads was 0.302 dm^2. This

is within the range of the surface area of sand reported by others (Burns, 1979). The values reported in table 1.1 of Burns et al. (1979) were 100x higher than measured values.

Anthracene was coated onto the surface of the 140 g glass beads in a roller bottle by addition of anthracene in dichloromethane. The roller bottle was rolled for two days to allow the dichloromethane to evaporate. To confirm coating of the glass beads, the roller bottle containing the glass beads was viewed under long wave U.V. light (Fig. 3.1). The beads that were coated with anthracene fluoresced a bright purple-white color under U.V light.

3.1.3 Extraction efficiency of anthracene from the glass beads

Eighty grams of salts medium was added to 140 g glass beads coated with 100 mg of anthracene and 8 samples were removed, dried with anhydrous Na_2SO_4 and Soxhlet extracted. The extracts were analyzed by GC and the extraction efficiency of the procedure was 52.57 ± 3.19 mg anthracene/ 140 g glass beads or about 53%.

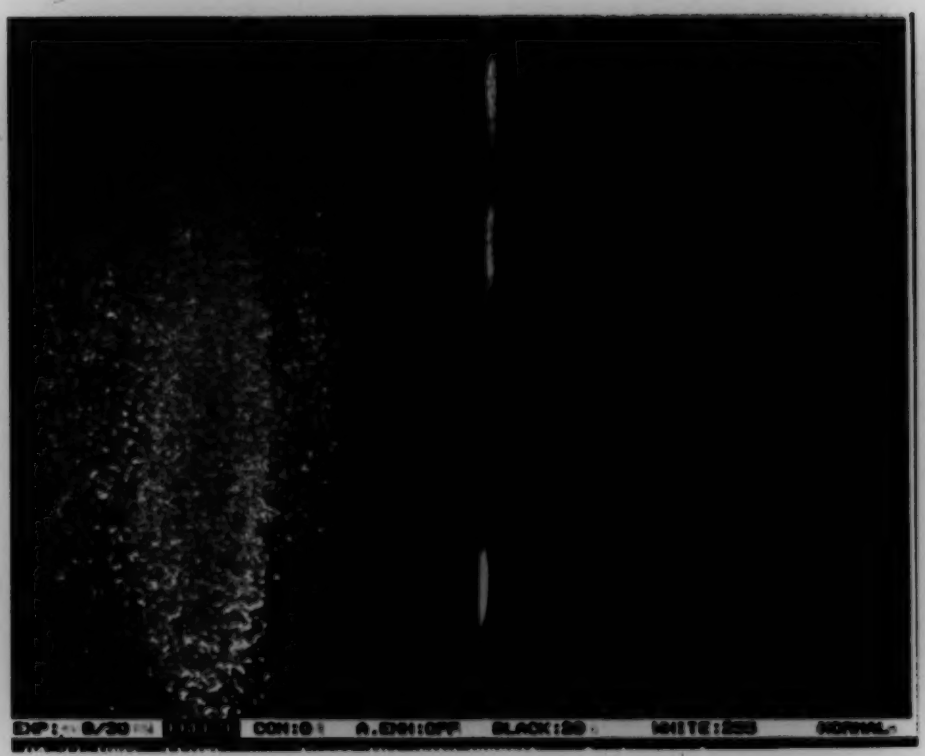
Chrysene was added as a surrogate standard at various stages of the procedure to determine where 47% of the anthracene was lost. Chrysene was added in dichloromethane directly to an extraction thimble containing Na_2SO_4-dried glass beads and the extraction procedure recovered 100% of the chrysene after 4 hours extraction. Chrysene, in dichloromethane, was also added to a sample that was dried with Na_2SO_4. Transfer of the contents to an extraction thimble resulted in 95% recovery of the surrogate. Thus, the extraction procedure was efficient at extracting close to 100% of the PAH after 4 hours.

Next, all materials which came in contact with anthracene during the procedure were extracted. During the coating process, the roller bottle itself was also coated with anthracene. The surface area of the roller bottle was 11.4% of the total surface area (glass beads and roller bottle surface area = 47.73 dm^2). However, 30 - 35% of the total

Figure 3.1 Effect of coating the glass beads with anthracene when viewed under ultraviolet light.

Anthracene dissolved in dichloromethane was added to glass beads in a roller bottle and rolled for 2 days to allow the dichloromethane to evaporate. In this case, the coated glass beads were then transferred to another roller bottle. The coated glass beads (a) were compared to non coated glass beads (b).

a b



anthracene added was extracted from an empty roller bottle. The reason for such a high proportion of anthracene coating the surface of the roller bottle was that crystals accumulated at the neck of the roller bottle. Therefore, most of the loss was due to the coating procedure in the roller bottle.

After samples were taken, the liquid was removed by filtering the sample through a 0.45 µm filter. Two filtrates were combined and extracted three times with dichloromethane and analyzed by GC. Less than 1% of the anthracene had passed through the filter.

To determine the removal of anthracene by bacteria from the glass beads only, the glass beads were coated with anthracene in a sterile roller bottle for solvent evaporation then transferred to a clean, sterile roller bottle. Since the exact amount of anthracene which was lost could not be determined, the proportion of anthracene degraded was calculated from zero time samples of the same roller bottle. It was found that about 80% of the added anthracene could be recovered from sterile samples between 1 and 7 days of rolling (Table 3.1), therefore, the actual removal rates were related to a sterile control treated identically.

Table 3.1. The efficiency of extraction of anthracene from sterile glass beads. All samples are compared to a sample taken before any aqueous medium was added (day 0). Triplicate samples were taken at each time point and analyzed by HPLC using naphthoic acid as an internal standard. The numbers in parentheses are the standard deviations of each sampling point.

Time (days)	% mean extraction efficiency (standard deviation)
0	99.0 (7.4)
1	79.0 (3.0)
2	86.3 (2.9)
4	73.7 (1.1)
7	78.0 (11.6)

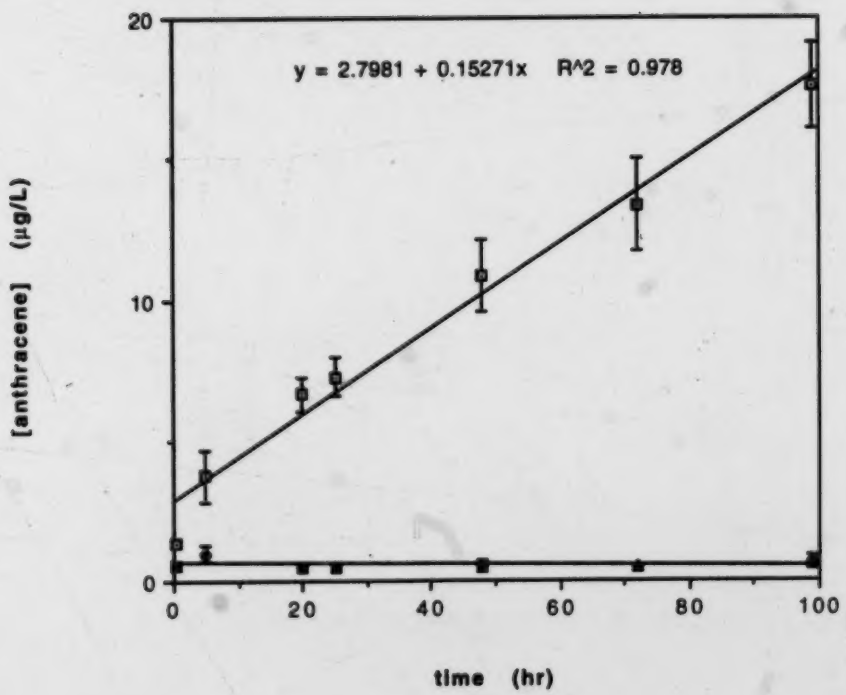
3.1.3.1 Effect of glass beads on the dissolution rate of anthracene

It was anticipated that coating glass beads with anthracene would increase the surface area of the anthracene exposed to the medium and thus the rate of anthracene dissolution when compared to the surface area of ground anthracene crystals in aqueous medium (Thomas et al., 1986). It has been predicted from other research that only the soluble fraction of PAHs can be degraded and therefore the rate of solubilization of PAHs determines the rate of growth of the bacteria (Stucki and Alexander, 1987). This may be the mechanism by which the soil system may increase the bacterial rate of transformation of anthracene when compared to the rate of degradation of crystals added to liquid culture.

To determine the effect that glass beads coated with anthracene had on the dissolution rate of anthracene, the following tests were performed in a scaled down version of the roller bottle. In the first experiment, [9-^{14}C]-anthracene was added to glass beads at a concentration which exceeded the saturation limit of anthracene in aqueous solution (40-70 µg/L, May et al., 1978) by two-fold (150 µg/L). To determine the effect of surface area on the rate of solubilization of anthracene, anthracene was added either as a small crystal at the bottom of a test tube without glass beads or to 17.5 g of glass beads in a test tube (total surface area = 5.3 dm^2) and minimal salts medium was added to both tubes. The proportion of minimal salts medium and glass beads in this test was identical to the ratio used in roller bottle experiments. The test tubes were rolled on a rolling apparatus at 3.5 rpm at 22°C. Samples were taken periodically, filtered through a 0.22 µm filter to remove particulate anthracene, and the filtrate was analyzed by liquid scintillation counting. When retrieving samples, care was taken not to remove any free anthracene crystals that were floating in suspension.

It was found that glass beads increased the rate of dissolution of anthracene dramatically over a four day period and the rate was constant as observed by the linear relationship in Figure 3.2. However, the final concentration in solution after 96 hours still

Figure 3.2 The effect of glass beads on the rate of dissolution of anthracene. [9-^{14}C]-anthracene was either added to glass beads in a roller tube (open squares) or added as a single crystal (closed diamonds) and rolled at 3.5 rpm and sampled periodically over 4 days. All samples were compared to a control. All test samples were taken in triplicate and error bars represent the standard deviation of each sampling point.


y = 2.7981 + 0.15271x R^2 = 0.978
[anthracene] (µg/L)
time (hr)
0
10
20
0
20
40
60
80
100

had not reached the solubility limit of anthracene in water (70 µg/L; May et al., 1978). When anthracene was added as a crystal with minimal surface area, the amount of anthracene in solution did not increase significantly throughout the sampling period.

To confirm that filtration was the best method for analyzing solubility, 1.5 mL samples were taken and centrifuged for five minutes at 11990 rpm to remove any crystals in solution. On average, the counts were 3 times higher than that found in the same sample after filtration (data not shown) suggesting that fine anthracene crystals do not sediment under these conditions.

3.1.3.2 Determination of the mechanism by which glass beads increase dissolution rate of anthracene

The previous experiment showed that the addition of glass beads increased the rate of solubilization of anthracene but it did not show the mechanism by which this increase occurred. To determine which factor may play the dominant role on the spontaneous dissolution rate of anthracene, a comparative experiment was performed.

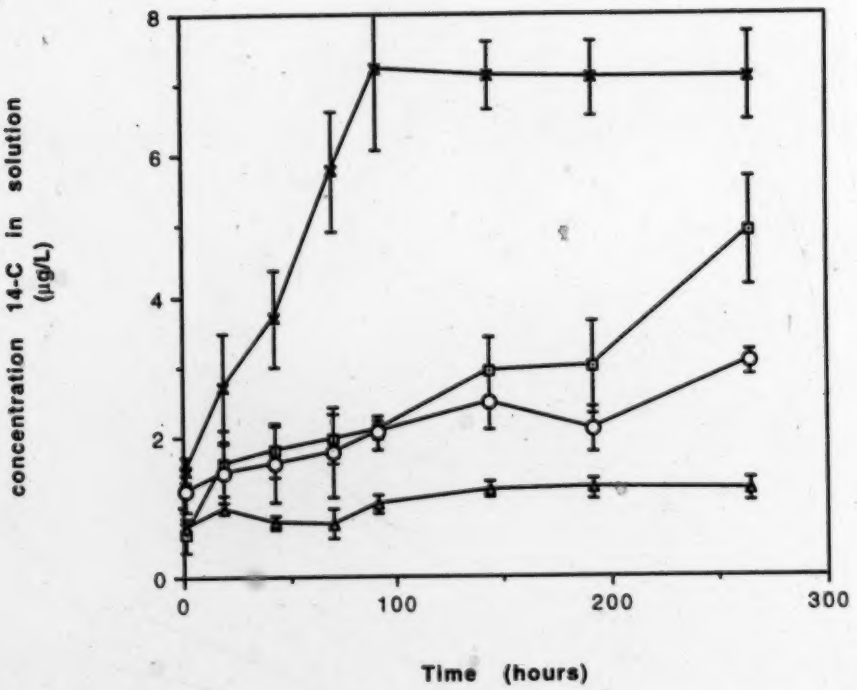
The total surface area of the test tube that came in contact with the medium was estimated to be 0.585 dm^2. Approximately 1.9 g of glass beads had the same surface area of the test tube. In this experiment three different samples were tested at two initial concentrations - at 2 times or 5 times the saturation limit of anthracene (Figs. 3.3a and 3.3b respectively). In the first case, the glass beads were coated with radioactive anthracene and were added to a test tube containing 10 mL salts medium. Only 3% of the radioactive anthracene was lost using the device in Figure 2.1 to transfer the labeled glass beads into the test tube at either initial concentration. In the second case, the inner surface area of the test tube, equivalent to 1.9 g glass beads, was labeled before the salts medium was added. Finally, the test tube inner surface was labeled and an equivalent surface area of unlabeled glass beads was added. As a control, a minimal surface area at the bottom of the test tube was labeled with ^{14}C-anthracene.

Figure 3.3 The effect of surface area and initial concentration of anthracene on the dissolution rate of anthracene.

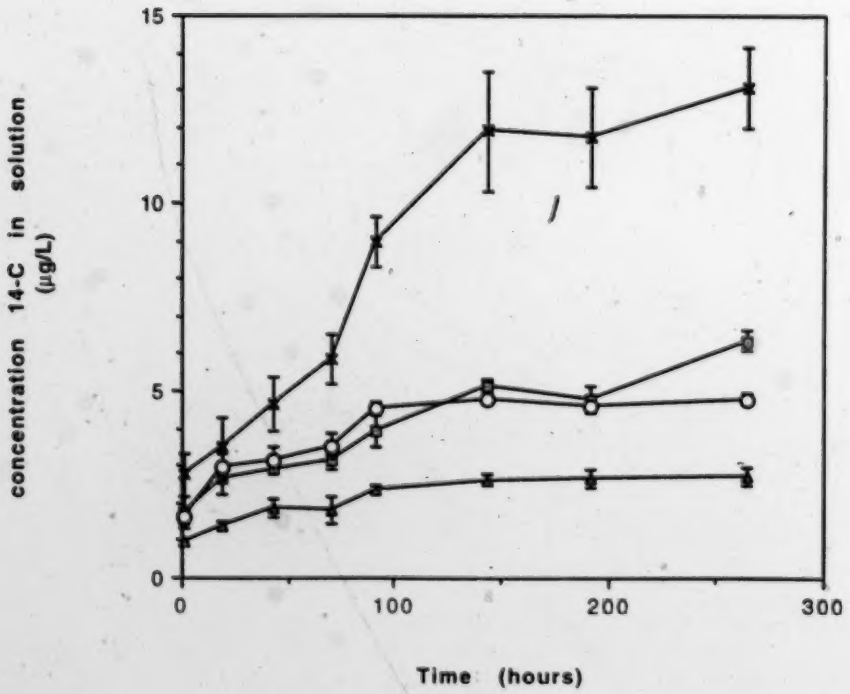
(a) 1.50 μg of [9-^{14}C]-anthracene was added either to 1.9 g glass beads (open squares) or an equivalent surface area of the test tube was labeled. To the labeled test tube, either glass beads were added (x) or not (open circle). As a control, a single crystal of radioactive anthracene was added to the bottom of an unlabeled test tube (open triangles). Samples were taken and passed through a 0.22 μm filter to remove particulate anthracene before samples were subjected to liquid scintillation. All tests were performed in triplicate and error bars represent the standard deviation of each sample.

(b) 5.00 μg of [9-^{14}C]-anthracene was added to samples as mentioned above. All treatments were represented by the same symbols as in (a).

A.


concentration 14-C in solution (µg/L)
0
2
4
6
8
0
100
200
300
Time (hours)

B.


concentration 14-C in solution (µg/L)
0
5
10
15
0
100
200
300
Time (hours)

The dissolution rate of anthracene was highest when the test tube was labeled and the unlabeled glass beads were added. When an equal surface area of the wall or glass beads were labeled, the dissolution rates were very similar but the effect of glass beads was slightly greater after 264 hours. This shows that surface area is the predominant factor affecting anthracene solubility in these two tests and mechanical disruption of anthracene by the glass beads exhibits only a minor role. These results did not appear to correlate with Fig. 3.2 -- the rate of dissolution in these experiments was lower. This may have been due to the low surface area that was coated with ^{14}C-anthracene.

The initial concentrations of anthracene added to each test tube also affected the solubility of anthracene in salts medium. When higher concentrations of anthracene were added, the initial rate of dissolution remained the same but the saturation level was higher even in samples where a single crystal of anthracene with a minimal surface area was added. Like the previous experiment the solubility limit of anthracene was never reached even at concentrations of anthracene up to 5x the the amount of anthracene required to saturate the solution.

3.2 Characterization and growth of cultures utilizing anthracene in roller bottles

Pure cultures used in these experiments were isolated from a mixed culture enriched on anthracene as the sole carbon and energy source. In soil contaminated with anthracene, this mixed culture was capable of degrading 600 mg anthracene/kg slurry within three days (Gray et al., 1994). The growth rate of this culture and the rate of removal of anthracene from the glass beads can be seen in Figure 3.4. The results show that the initial concentration of the slow growers outnumber those of the fast growers by 10:1 and that concentrations of viable cells in each increased at approximately the same rate (fig. 3.4a). Also, the removal rate of anthracene by this culture under these conditions was approximately 400 mg anthracene/kg glass bead slurry in 7 days. After this initial burst of

Figure 3.4 Growth of a mixed culture enriched on anthracene as the sole carbon and energy source.

(a) Viable counts of the slow growing, gram positive subpopulation (solid diamonds) and the fast growing, gram negative subpopulation (open squares) of a mixed culture isolated from creosote contaminated soil and enriched on anthracene.

(b) Degradation of anthracene by this mixed culture over 14 days. The mixed culture was added to a roller bottle containing anthracene coated glass beads at either a 5% (%v/v) inoculum (x) or 25% inoculum (open triangles). Each test represents the average of duplicate samples. Removal of anthracene from a sterile roller bottle representing any abiotic effects on anthracene during the experiment was also monitored (open squares).

A.

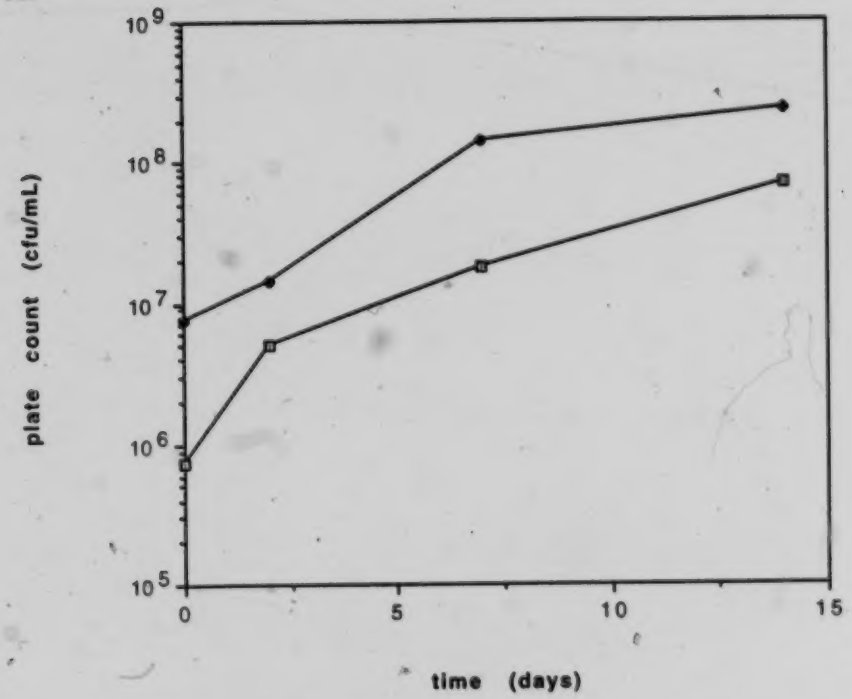
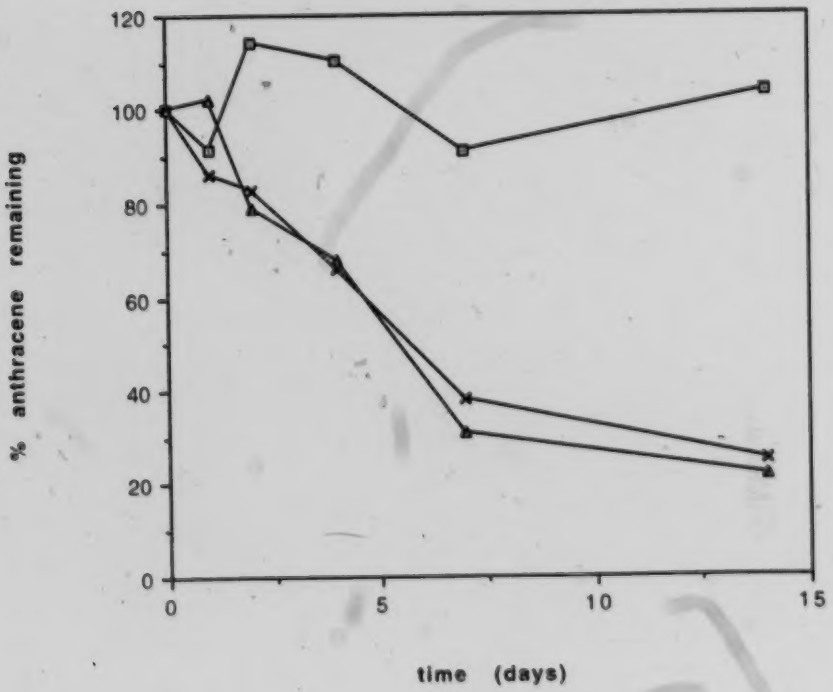


plate count (cfu/mL)
10^9
10^8
10^7
10^6
10^5
0
5
10
15
time (days)

B.


% anthracene remaining
120
100
80
60
40
20
0
0
5
10
15
time (days)

activity in the first week, the levels of anthracene in the roller bottle plateaued with approximately 20% of the anthracene remaining (corresponding to 100 mg anthracene/kg glass beads; Fig. 3.4b). The initial concentration of the mixed culture which was inoculated into the roller bottle affected neither the rate nor the extent of anthracene degradation in the roller bottle after 7 days incubation.

3.2.1 Ability of an isolate from the slow growing population to utilize anthracene

The mixed culture appeared to consist of two sub-populations as previously described. From the slow growing population, one organism was isolated. *Rhodococcus* S1, which was previously characterized by Tongpim and Pickard (1996). This organism was capable of utilizing anthracene as a sole carbon and energy source (Fig. 3.5). It was able to remove approximately 40% of the anthracene on glass beads in 7 days (200 mg/kg glass beads). Over the first four days of the incubation, anthracene was not removed. However, viable counts increased 400 fold during this time. After viable counts reached 10^8 cfu/mL, significant removal of anthracene began. Similar to the mixed culture results, anthracene levels in the roller bottle plateaued after 7 days.

To determine if the cells preferred a site of attachment for growth, viable counts of the glass beads were compared to counts of the aqueous medium. Viable cells from the glass beads outnumbered the bacteria in the medium by nearly 10 fold ($2x10^8$ cfu/g removed from glass beads vs. $3.0x10^7$ cfu/mL in aqueous phase). Viable counts in the aqueous medium were more reproducible than viable counts from the glass beads.

3.2.2 Characterization of the isolate from the fast growing population

The other isolate used to determine interactions with *Rhodococcus* S1, came from the fast growing gram negative population. This isolate was a motile, gram negative rod.

Figure 3.5 Growth curve of *Rhodococcus* S1 on anthracene coated glass beads in a roller bottle.

Ability of a gram positive, slow growing member of the enrichment mixed culture previously identified as *Rhodococcus* S1 to grow in the presence of anthracene as the only carbon and energy source (open squares; cfu/mL). Degradation of anthracene was detected as the disappearance of the parent compound (closed diamonds). The plotted values represent the mean of duplicate roller bottles

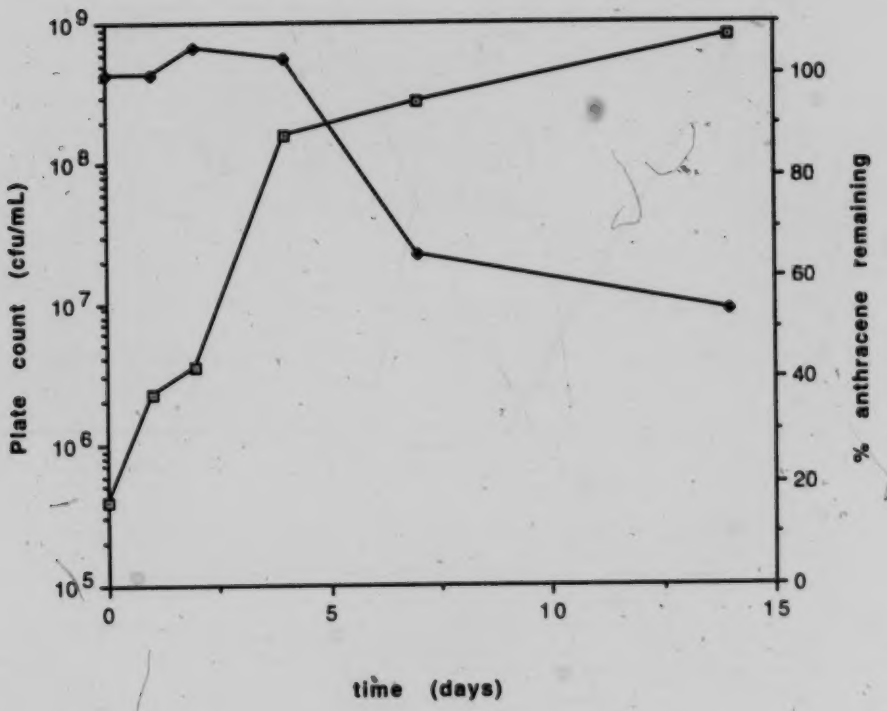


Plate count (cfu/mL)
10^9
10^8
10^7
10^6
10^5
% anthracene remaining
100
80
60
40
20
0
0
5
10
15
time (days)

Table 3.2 Physical and biochemical characteristics of Pseudomonas isolate G1

Characteristic	Reaction
Bacteriological tests	
Motility	+
Gram reaction	-
Morphology	Rods
Biochemical tests	
ß-galactosidase	-
Arginine dihydrolase	+
Lysine decarboxylase	-
Ornithine decarboxylase	-
H_2S production	-
Urease	-
Tryptophan metabolism	-
Voges Praskauer	-
Gelatin liquefaction	-
Oxidase	+
Nitrate reduction	±
Growth tests	
glucose	-
mannitol	-
inositol	-
sorbitol	-
rhamnose	-
sucrose	-
melibiose	-
amygdalin	-
arabinose	+
citrate	+

It was characterized to the genus level of *Pseudomonas* and designated G1 (Table 3.2). Initially, it appeared that this organism could grow on anthracene as its sole carbon and energy source. However, after successive transfers the ability to remove anthracene was lost (Fig. 3.6). Interestingly, when the culture was transferred to a new culture flask, viable counts increased exponentially. However, each successive exponential growth spurt decreased with each transfer and overall viable counts decreased.

It was suspected that this organism may require additional nutrients that were supplied by the mixed culture in order to utilize anthracene . So the culture was supplemented with a source of B vitamins by adding yeast extract at 0.2 g/L. The B vitamins are important cofactors required for the degradation of PAHs (Sanseverino et al., 1993). However, in the presence of yeast extract, this culture may have used the yeast extract as a carbon and energy source and thus was still unable to metabolize anthracene. Likewise, the addition of low concentrations of salicylate (0.005%), used to induce enzymes involved in the NAH pathway (Ogunseitan et al., 1991), did not stimulate growth of *Pseudomonas* G1 on anthracene. Therefore, the mixed culture may have provided *Pseudomonas* G1 with some other nutrients or metabolites from anthracene in order to be maintained within this community. Alternatively, contaminants in the anthracene added to the medium, such as phenanthrene, may have served as a growth substrate.

3.2.3 Isolation of another slow growing bacterium from the same soil

Another microorganism, A1, was isolated from the same original creosote contaminated soil but it was not found in the anthracene enrichment culture. This organism was a gram positive, acid-fast coccus, capable of degrading pyrene, anthracene, phenanthrene and naphthalene from minimal medium agar, when these PAHs were sprayed as a thin layer on the surface of the agar. This was observed by zones of clearing around the colonies. The degradation curve of Gram positive A1 (Fig. 3.7) shows similar results

Figure 3.6 Growth curve of the Gram negative isolate *Pseudomonas* G1 on anthracene coated glass beads in a roller bottle.

Growth profile as determined by the viable cells in the aqueous medium of the roller bottle (open squares) compared to the degradation of anthracene in that roller bottle (open circles). Degradation of anthracene was compared to a sterile control treated identically which represented 100% anthracene remaining. After 7 days, G1 was transferred to a new sterile roller bottle as a 10% inoculum. Viable counts represent the average of triplicate samples whereas anthracene concentrations represent the average of duplicate roller bottles treated identically.

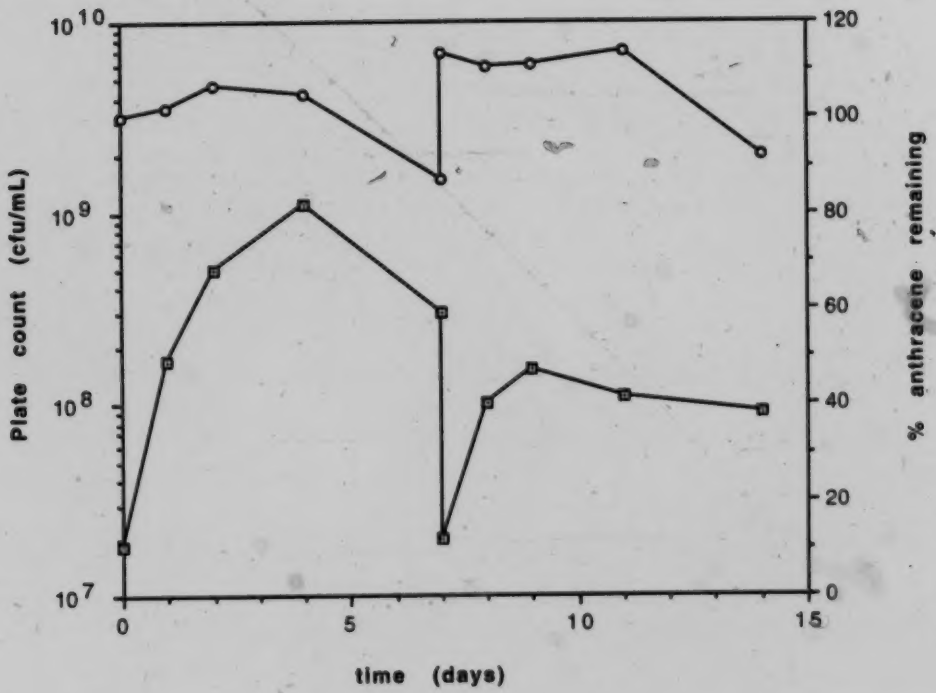
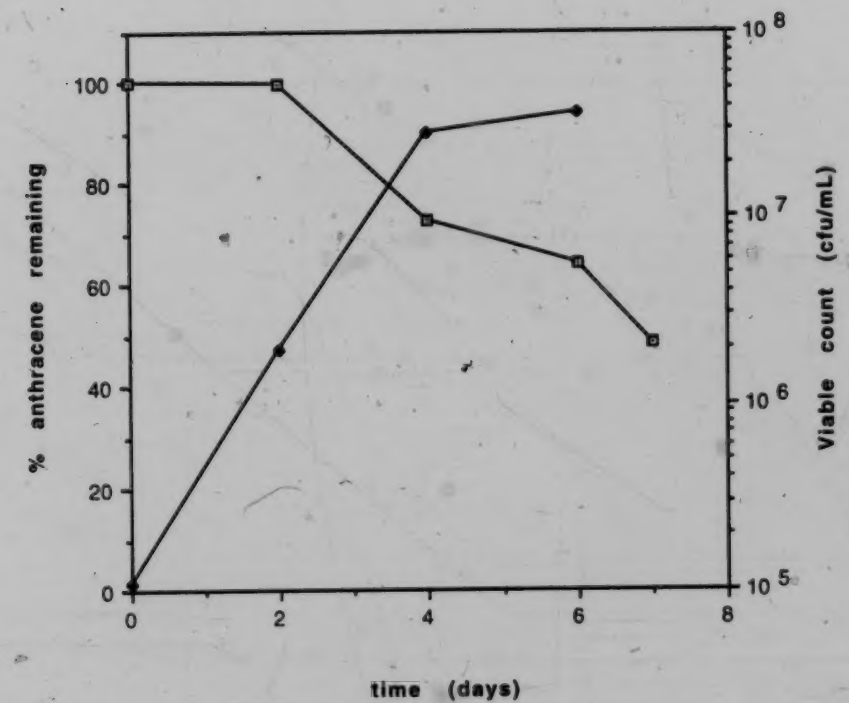


Plate count (cfu/mL)
10^{10}
10^{9}
10^{8}
10^{7}
% anthracene remaining
120
100
80
60
40
20
0
0
5
10
15
time (days)

Figure 3.7 Growth curve of Gram positive isolate A1 on anthracene coated glass beads in a roller bottle rolled at 3.5 rpm.

Growth profile of isolate A1 determined by viable counts in the aqueous medium in the roller bottle (closed diamonds) compared to the degradation of anthracene in that roller bottle (open squares). Viable counts represent the average of triplicate samples whereas anthracene concentrations represent the average of duplicate roller bottles.


% anthracene remaining
Viable count (cfu/mL)
time (days)
100
80
60
40
20
0
0
2
4
6
8
10^8
10^7
10^6
10^5

to *Rhodococcus* S1 - about 50% of the anthracene was removed in 7 days. In this case, the lag phase, observed before anthracene removal began, was only 2 days. Also, bacterial numbers were an order of magnitude lower as viable bacteria in the aqueous medium only were counted.

3.3 Comparison of anthracene removal by *Rhodococcus* S1 in the roller bottle and shake flask

Before investigating the interactions of *Rhodococcus* S1 with anthracene in the roller bottle environment, the rate of removal of anthracene in the roller bottle was compared to shake flasks with coils that break up anthracene crystals (Fig. 3.8). The overall removal of anthracene was similar in both systems. However, the rates of removal were different. In the shake flask, there was a continuous linear decrease of anthracene over the seven day incubation. However, in the roller bottle, there appeared to be at least a four day lag period before a more rapid rate of degradation of anthracene began.

3.3.1 Acclimation of *Rhodococcus* S1 to the roller bottle environment

The lag time before degradation of anthracene began in the roller bottle environment was consistent in *Rhodococcus* S1. There were several reasons why this lag period may have occurred and each hypothesis was tested. First, *Rhodococcus* S1 may have required time to acclimate itself to the glass bead environment. Inoculum for *Rhodococcus* S1 always came from a shake flask with a 2-3 week old culture. Therefore, *Rhodococcus* S1 was inoculated from a shake flask into a roller bottle culture (preacclimated). After this culture was incubated for 7 days, it was transferred to another roller bottle (acclimated). The amount of anthracene degraded was compared between preacclimated and acclimated cultures in roller bottles (Fig. 3.9). In the preacclimated culture, *Rhodococcus* S1 had a five day lag time before significant degradation of anthracene began. Upon transfer to a

Figure 3.8 Degradation of anthracene by *Rhodococcus* S1 compared in two different systems.

The ability of *Rhodococcus* S1 to degrade anthracene in shake flasks (solid circles) and the roller bottle (open squares) over 7 days. All samples were tested in duplicate and compared to sterile controls treated identically.

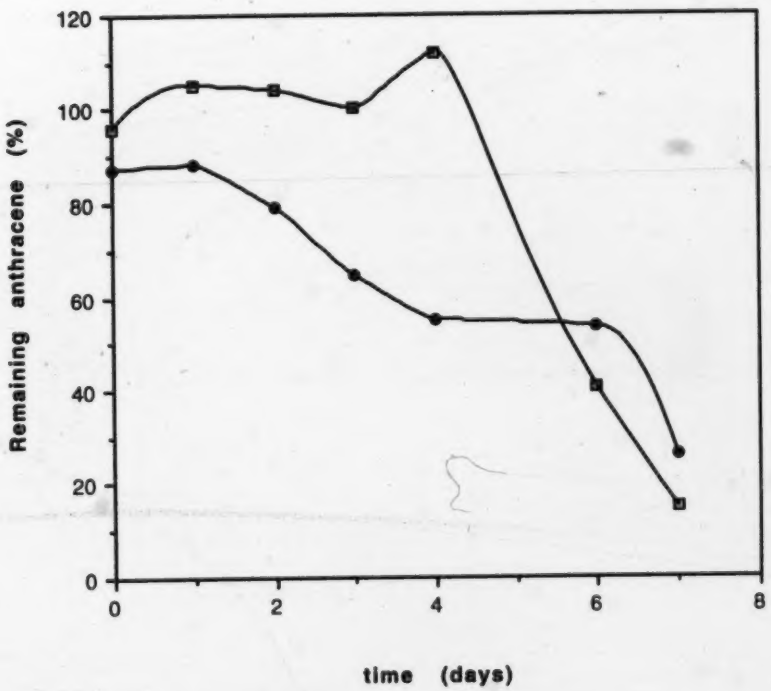
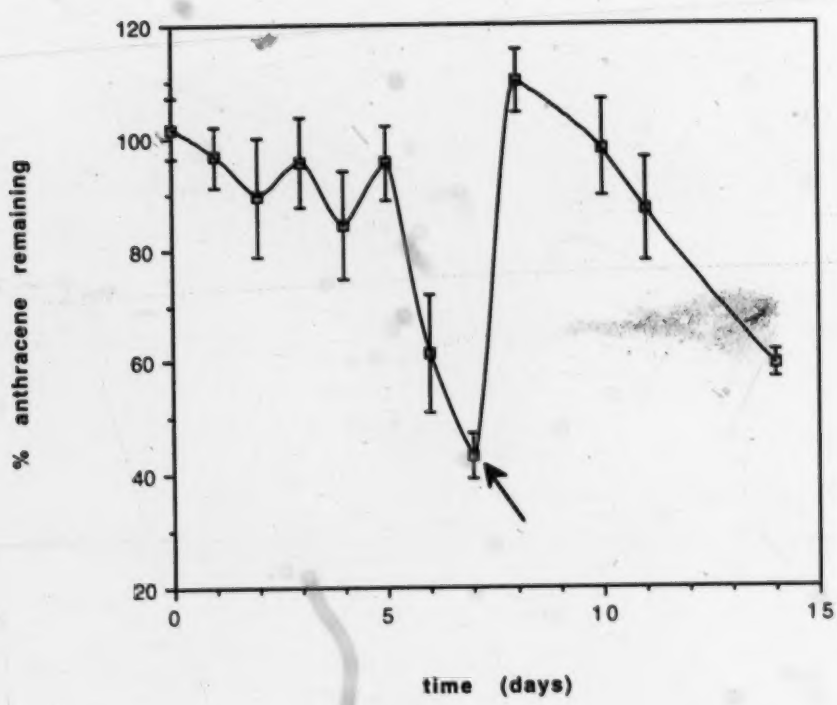

120
100
80
60
40
20
0
0
2
4
6
8
Remaining anthracene (%)
time (days)

Figure 3.9 The effect of acclimatizing *Rhodococcus* S1 to the roller bottle system. *Rhodococcus* S1 was grown in the presence of anthracene as the sole carbon source and the extent of degradation was followed. After day 7, the culture was transferred to another sterile roller bottle (as shown by arrow on the graph) containing 100 mg anthracene/140 g glass beads and degradation was followed. The error bars represent the standard deviation of samples taken from three roller bottles.


120
100
80
60
40
20
0
5
10
15
% anthracene remaining
time (days)

new roller bottle, the lag time exhibited by the acclimated culture decreased and significant removal of anthracene began after 2 days. But, the overall rate of anthracene removed during the 7 day incubation did not increase after the acclimation period.

3.3.2 Effect of physical factors on the growth of *Rhodococcus* S1 on anthracene

It has been shown in previous reports that the solubility of PAHs determine their rate of removal (Bossert and Bartha, 1986). Therefore, the low aqueous initial concentration of anthracene and low rate of dissolution may not be enough to allow for the growth of *Rhodococcus* S1 thereby explaining the lag time.

To test this hypothesis, a roller bottle containing sterile, coated glass beads was rolled for 5 days to allow anthracene levels to increase and equilibrate. *Rhodococcus* S1 was then added to the roller bottle and the rate of removal of anthracene was followed. In this experiment, the lag time decreased from 5 days in preacclimated cultures to 3 days in acclimated cultures (fig. 3.10). Again, overall degradation of anthracene reached the same level in both cultures. So this factor alone did not account for the full lag time that *Rhodococcus* S1 experienced when inoculated into a roller bottle environment but together with preacclimated cultures it approaches the rate found in whole soil systems.

3.3.3 Colonization of glass beads and anthracene crystals by *Rhodococcus* S1

Tongpim and Pickard (1996) have previously shown that the hydrophobic membrane of *Rhodococcus* S1 allows this organism to attach directly to hydrophobic surfaces such as an anthracene crystal. Therefore, a lag time may have occurred to allow *Rhodococcus* S1 to attach to either the glass bead or anthracene crystals before significant losses in anthracene were observed. The site of preferential attachment of *Rhodococcus* S1 was observed using SEM (Fig. 3.11). Initially, the preparation of samples required

Figure 3.10 The effect of anthracene equilibrium dynamics on the ability of *Rhodococcus* S1 to degrade anthracene.

Sterile medium was added to a sterile roller bottle and rolled for 5 days before inoculating the bottle with *Rhodococcus* S1. The extent of degradation of anthracene in this system (open squares) was compared to a roller bottle which was inoculated immediately with *Rhodococcus* S1 (closed diamonds). Error bars represent the standard deviation of samples taken from three identical roller bottles.

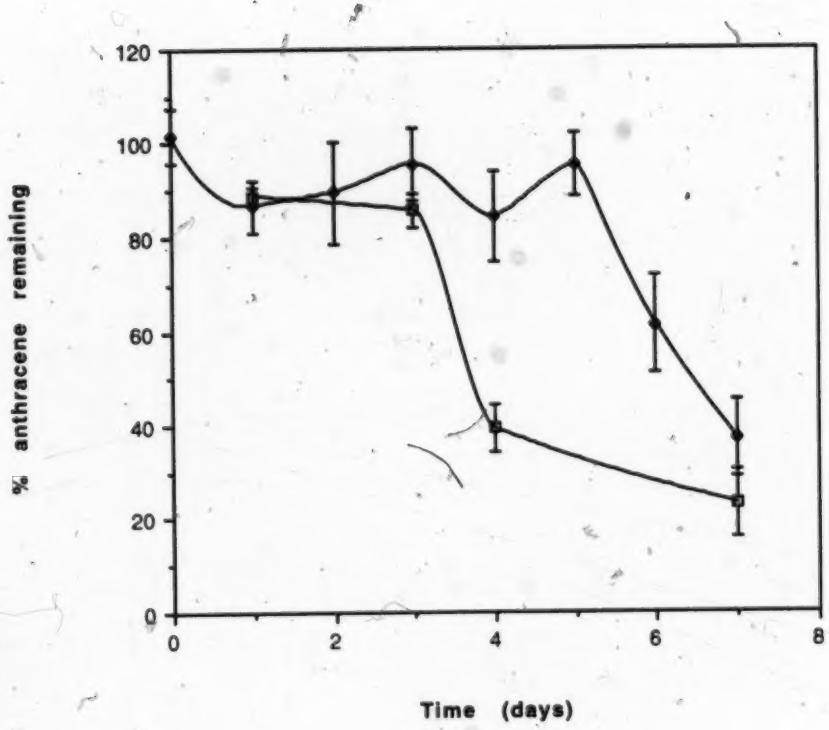

% anthracene remaining
120
100
80
60
40
20
0
0
2
4
6
8
Time (days)

Figure 3.11 Colonization of anthracene crystals and glass beads by Rhodococcus S1.

(a) Scanning electron micrograph of an anthracene crystal on a glass bead. This micrograph shows that the crystals only stay in indentations in the glass bead away from abrasion by other glass beads.

(b) A magnified view of (a) showing the absence of bacteria on the crystal after S1 was incubated for four days.

(c) Colonization of S1 on the glass bead. Colonization of the glass bead only occurred on the glass bead which were protected from abrasion by other glass beads.

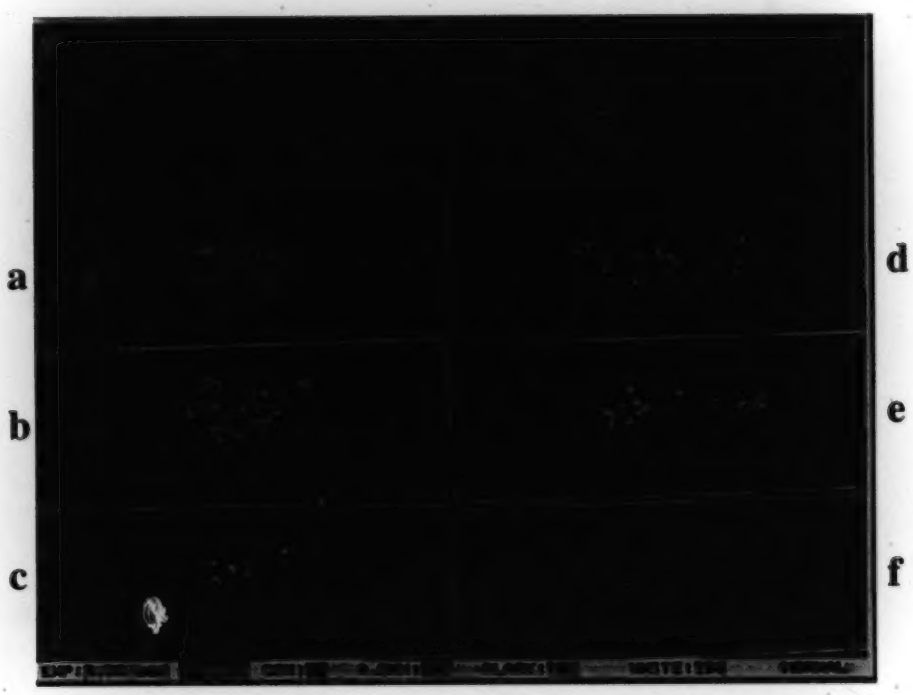
(d) Colonization of an anthracene crystal by S1 after 14 days incubation. This micrograph shows an anthracene crystal sloughed off the glass beads and clumped together with other crystals to form an aggregate.

a b

c d

Figure 3.12 The effect of ethanol concentrations on the proportion of anthracene that stays on the glass beads.

Ethanol was used as a post fixative wash to remove salts and water from samples prepared for SEM. The effect of increasing the ethanol content in solution on the removal of anthracene from the glass beads was observed under long wave ultraviolet light. Fluorescence from the aromatic rings of anthracene were followed in samples where glass beads were filtered to remove water (a), washed with water (b), washed with 30% ethanol for 15 min (c), washed with 50% ethanol for 12 min (d), washed with 90% ethanol for 2 min (e) and washed with 100% ethanol for 2 min (f). All slides taken represent cumulative effects. For example the 90% wash with ethanol was previously washed with 50% ethanol (12 min), 30% ethanol (15 min), washed with water and filtered.


a
b
c
d
e
f

removal of salts and dehydration of the sample by increasing incremental washes with ethanol. Absolute ethanol was initially used as the final wash to completely dehydrate the glass beads. Figure 3.12 shows the effect of ethanol washes on the dissociation of anthracene from the glass beads. A wash with 88% ethanol had little effect on the removal of anthracene from the glass beads while absolute ethanol removed all the anthracene from the glass beads. To prevent the loss of anthracene from the glass beads, samples were washed with no more than 90% ethanol. Then the sample was air dried and viewed under SEM after coating with gold.

After one to two days, most of the anthracene present on the smooth surfaces of the glass beads had sloughed off (data not shown). As a result it appeared that anthracene did not coat the glass beads uniformly and that crystals stayed attached to the glass beads only if they were protected from the smooth surface by collecting in clefts of the bead (Fig. 3.11a and b).

The pattern of colonization seen for *Rhodococcus* S1 was similar to the locations of anthracene crystals (Fig. 3.11c). That is, small colonies of *Rhodococcus* S1 were found attached to the glass beads in places protected from abrasion by other glass beads.

Anthracene crystals which were sloughed off from the smooth surface of the glass beads were colonized by large quantities of *Rhodococcus* S1 (Fig. 3.11d). Large indentations were formed on the crystal within the vicinity of each degrading bacterium. It appeared that *Rhodococcus* S1 bound to the surface of the anthracene crystal by association with its hydrophobic cell wall. Here it degraded the anthracene by either releasing a solubilizing substance onto the crystal or taking up anthracene as it was solubilized from the crystal.

It was difficult to monitor this colonization in a temporal manner because of low initial cell counts and the length of time that was required for *Rhodococcus* S1 to find and attach to a crystal. But it was confirmed that colonization plays an important role in facilitating removal of anthracene from this system.

3.4 Mixed culture studies

The original mixed culture was able to remove anthracene from glass beads at about 50 mg·kg glass beads^{-1}·day^{-1} (Fig. 3.4b). A pure culture of *Rhodococcus* S1 could only remove anthracene at half this rate (Fig. 3.5). Further, *Pseudomonas* G1 was unable to utilize anthracene as a sole carbon source (Fig. 3.6). Thus, it followed that interactions between species in the community were likely the cause of the enhanced ability of the mixed culture to degrade anthracene.

Since in the orginal mixed culture there were 2 sub-populations in the community, it seemed feasible to study the interactions of *Rhodococcus* S1 with one other member of the fast growing population, G1. For comparison, the interaction of *Rhodococcus* S1 with one member of the original soil community, Gram positive A1, was also followed.

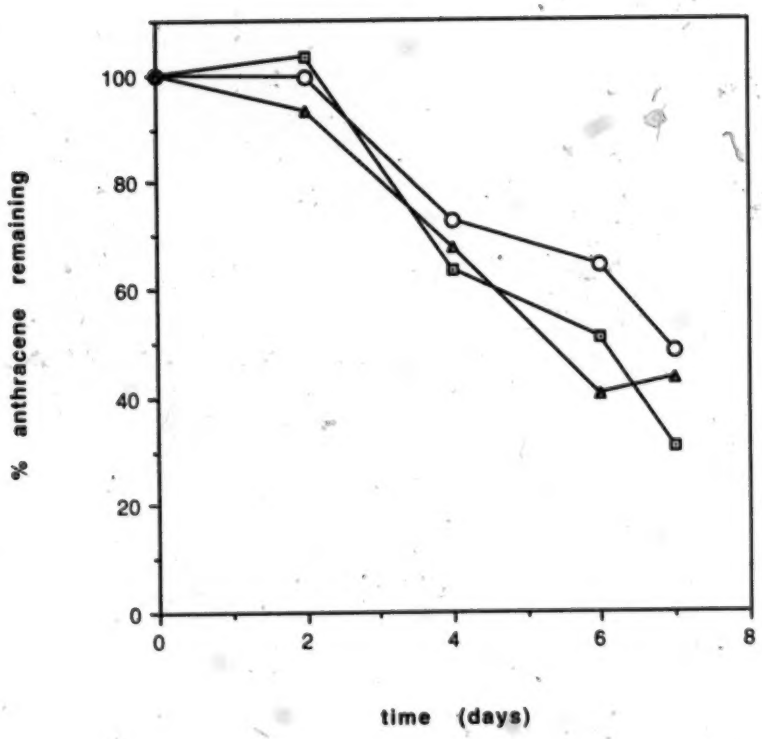
The gram positive, slow growing isolate, A1, was selected because it was easy to differentiate from *Rhodococcus* S1 on agar plates - the colonies of A1 were a bright orange color while *Rhodococcus* S1 was white. The interaction of the fast growing *Pseudomonas* G1, with *Rhodococcus* S1 was selected for these tests to attempt to discover why this organism was capable of existing in a mixed culture enriched on anthracene as a sole carbon and energy source even though it could not degrade anthracene.

3.4.1 Interactions within a coculture of two slow growing isolates

Rhodococcus S1 was incubated in a shake flask along with an equal ratio of A1 over three monthly transfers to establish a stable coculture. After three weeks growth of this stable culture, viable cells were counted and it was found that *Rhodococcus* S1 outnumbered Gram positive A1 by a 3:1 ratio. The roller bottles were inoculated with this stable coculture and removal of anthracene from glass beads was followed for seven days (Fig. 3.13). Throughout the entire duration of this experiment, the ratio of S1:A1 remained

Figure 3.13 The effect of a slow growing acid-fast microorganism on the ability of *Rhodococcus* S1 to remove anthracene from the roller bottle system.

The extent of degradation of anthracene in the roller bottle was followed when *Rhodococcus* S1 and the gram positive, acid fast isolate, A1 were grown together in a stable mixed culture (open square) and compared to the abilities of pure cultures of either S1 alone (open triangles) or A1 alone (open circles) to degrade anthracene. Each point represents the average amount of degradation of anthracene in duplicate samples.


% anthracene remaining
100
80
60
40
20
0
0
2
4
6
8
time (days)

the same demonstrating that a stable mixed culture was achieved. The results in Figure 3.13 revealed that the extent of anthracene degradation after 7 days incubation in the pure cultures was equivalent to that in the mixed culture - all cultures degraded approximately 50% of the total anthracene present. Even the rates of degradation by both the pure cultures and the mixed culture were similar.

However, the viable counts of each of the pure cultures was equivalent to the viable counts of the coculture. This demonstrated that there is a competitive interaction affecting the growth of both organisms in a stable coculture (Table 3.3). The initial cell density of each isolate was greater in the coculture than in pure cultures. In all the samples, the cell density after 7 days growth ranged from $3.7x10^7$ to $6.6x10^7$ cfu/mL in the aqueous phase. Thus, the rate of solubilization of anthracene appeared to support the growth of up to $7x10^7$ cells/mL even in cocultures.

Table 3.3. The effect of a coculture on the viable cell count of each member in the community compared to the pure culture components. All values are in colony forming units/mL (cfu/mL) in the liquid portion of the culture. The bold lettering in parenthesis refers to the viable counts of that isolate in the coculture. Viable counts are the average of three replicates.

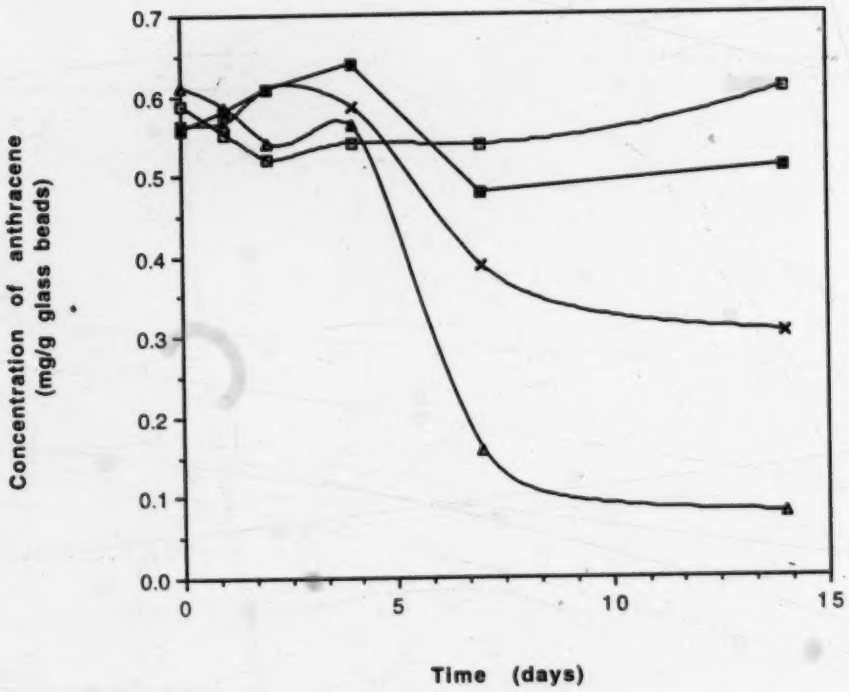
time (days)	A1 pure culture	S1 pure culture	**[A1]**+S1 coculture	A1+**[S1]** coculture
0	$2.7x10^5$	$4.0x10^5$	$4.8x10^5$	$1.3x10^6$
2	$2.3x10^6$	$1.4x10^6$	$3.3x10^6$	$9.9x10^6$
4	$2.8x10^7$	$7.2x10^7$	$1.6x10^7$	$4.6x10^7$
6	$3.7x10^7$	$6.6x10^7$	$1.8x10^7$	$4.4x10^7$

3.4.2.1 Determination of the interactions between *Rhodococcus* S1 and a fast growing *Pseudomonas* G1

Three experiments were set up to determine the interaction between *Rhodococcus* S1 and *Pseudomonas* G1. In the first set, *Rhodococcus* S1 was added to the roller bottle

Figure 3.14 The effect of a gram negative isolate, *Pseudomonas* G1, on the ability of *Rhodococcus* S1 to degrade anthracene - situation 1.

The capability of pure cultures of *Rhodococcus* S1 (x) and *Pseudomonas* G1 (solid squares) to degrade anthracene were compared to a coculture of these two isolates (open triangles). Removal of anthracene by abiotic factors was monitored by sampling a sterile control (open squares). Because *Pseudomonas* G1 was inoculated into roller directly from a stock suspension containing glycerol, approximately 20 mg of glycerol was added to cultures in which *Pseudomonas* G1 was introduced.


Concentration of anthracene
(mg/g glass beads)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
5
10
15
Time (days)

from a shake flask to an initial concentration of $4x10^5$ cells/mL. *Pseudomonas* G1 was added in 0.1 mL of 20% glycerol stock to the roller bottle to an initial concentration of $1.5x10^7$ cells/mL. The ability of this coculture to degrade anthracene is presented in Figure 3.14. *Rhodococcus* S1 was capable of degrading 40% of the anthracene when in a pure culture and *Pseudomonas* G1 was unable to degrade significant amounts of anthracene. However, when the two organisms were mixed, into a coculture, 80% of the anthracene was degraded within 7 days.

Viable cell counts of *Rhodococcus* S1 in the mixed culture were unable to be initially determined due to the large excess of *Pseudomonas* G1 at the beginning. But after 7 days, *Rhodococcus* S1 cell numbers exceeded *Pseudomonas* G1 numbers by nearly two times ($4.8x10^8$ cfu/mL S1 and $2.6x10^8$ cfu/mL G1).

While this experiment was inadvertantly flawed due to the addition of an alternate carbon source, these results suggest that some factor in this mixed culture was enhancing the degradation of anthracene. This increase in anthracene degradation rates could have been due to a positive interaction between the two organisms or it could be have been due to the addition of 20 mg of glycerol when *Pseudomonas* G1 was added to the roller bottle. To determine if the enhanced degradation rates were due only to the interaction of the coculture, no alternate carbon source was added in the following two experiments.

3.4.2.2 Degradation of anthracene by a coculture with similar proportions to the original mixed culture

The original mixed culture from which both *Rhodococcus* S1 and *Pseudomonas* G1 were isolated from consisted of approximately 100 Gram positive organisms for every Gram negative microbe. To simulate this circumstance, *Rhodococcus* S1 was added in a 20 fold excess compared to *Pseudomonas* G1. Initial concentrations of each organism were determined by direct counts of shake flasks of each pure culture. Both *Rhodococcus* S1 and *Pseudomonas* G1 were added to the roller bottle from shake flasks incubated for

two weeks. Initial cell concentrations of *Rhodococcus* S1 and *Pseudomonas* G1 were $3.9x10^5$ cfu/mL and $2x10^4$ cfu/mL, respectively.

Final concentrations of anthracene were the same for both roller bottles which contained *Rhodococcus* S1 whereas no loss of anthracene was measured in the pure culture of *Pseudomonas* G1 (Fig. 3.15). After day 7, cell densities of *Rhodococcus* S1 were equivalent to cell densities of *Pseudomonas* G1. In the pure cultures, *Rhodococcus* S1 levels were only 2-fold higher than the levels in the coculture. The cell concentrations of *Pseudomonas* G1 in the coculture exceeded levels in the pure culture by 30 times ($4.0x10^7$ and $1.4x10^6$ cfu/mL, respectively). These results imply that this coculture had no effect on the ability of *Rhodococcus* S1 to degrade anthracene. However, the coculture does have a positive effect on the survival of *Pseudomonas* G1.

3.4.2.3 Establishing a coculture of *Rhodococcus* S1 and *Pseudomonas* G1

To establish a stable coculture of *Rhodococcus* S1 and *Pseudomonas* G1, both organisms were incubated together in a shake flask containing anthracene as the sole carbon and energy source for 14 days. This culture was transferred to fresh medium with anthracene and incubated for a further 14 days. This procedure was repeated once more before the culture was considered stable. At this point, *Pseudomonas* G1 cell concentrations exceeded *Rhodococcus* S1 concentrations by about 20 times ($3x10^5$ S1/mL and $6.6x10^6$ G1/mL). Viable counts beyond the beginning of the experiment were difficult to determine due to the fast growth of *Pseudomonas* G1 which masked the growth of *Rhodococcus* S1. Even though *Rhodococcus* S1 could not be detected, this culture was considered a coculture because *Pseudomonas* G1 was capable of survival on anthracene as the only carbon and energy source over several transfers while a pure culture of *Pseudomonas* G1 was unable to grow in these conditions.

Figure 3.15 The effect of *Pseudomonas* G1 on the ability of *Rhodococcus* S1 to degrade anthracene - situation 2.

A coculture containing 20 fold more *Rhodococcus* S1 than *Pseudomonas* G1 was inoculated into roller bottles and the ability of this coculture (open squares) was compared to the extent of degradation of anthracene by pure cultures of either *Rhodococcus* S1 (x) or *Pseudomonas* G1 (open triangles). A sterile control (open circles) was used to monitor extraction efficiency of anthracene from the glass beads. All samples were tested in duplicate.

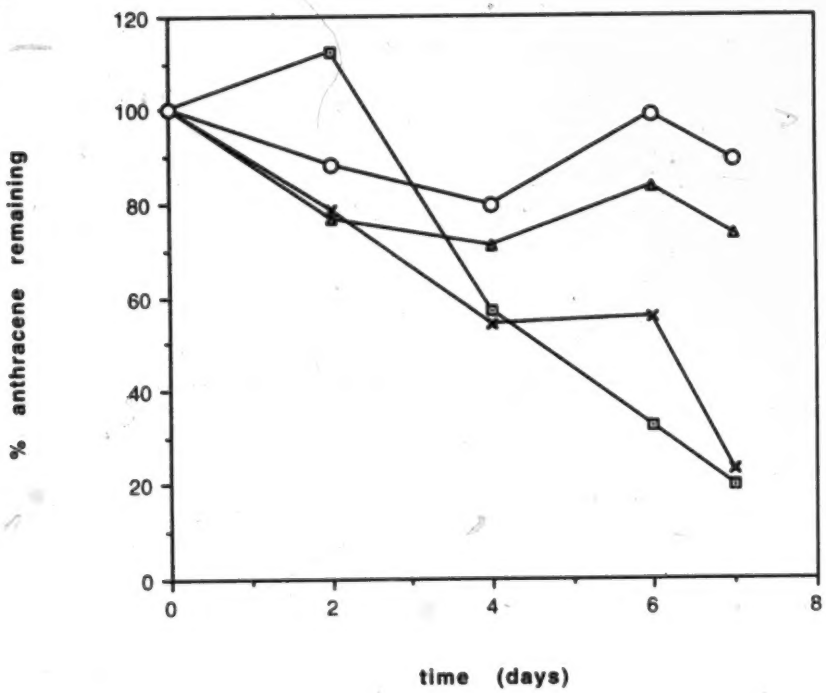
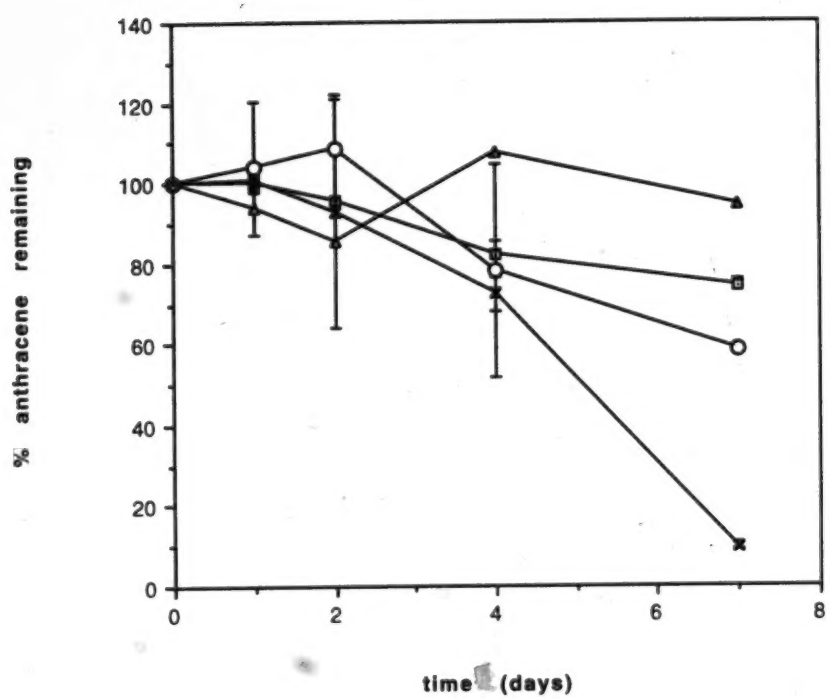

120
100
80
60
40
20
0
0
2
4
6
8
% anthracene remaining
time (days)

Figure 3.16 The extent of removal of anthracene by a stable coculture of *Rhodococcus* S1 and *Pseudomonas* G1 - situation 3.

A stable coculture of *Rhodococcus* S1 and *Pseudomonas* G1 was chosen arbitrarily as the third monthly transfer of this coculture. The degradation of anthracene by this stable coculture (open circles) was compared to the degradation of anthracene by its component pure cultures, *Rhodococcus* S1 (x) and *Pseudomonas* G1 (open squares). The extraction of the sterile control is represented by the open triangles. All timepoints represent the average of three roller bottles treated identically. Error bars represent the standard deviation of each timepoint.


140
120
100
80
60
40
20
0
0
2
4
6
8
% anthracene remaining
time (days)

This stable coculture was inoculated into a roller bottle and analysis of the loss of anthracene in the mixed culture was compared to levels of anthracene degraded by pure cultures (Fig.3.16). Figure 3.16 shows that the mixed culture was capable of degrading significantly more anthracene than the *Pseudomonas* G1 pure culture over the course of the experiment. Further, the pure culture of *Rhodococcus* S1 was capable of degrading significantly more anthracene than the coculture.

Thus, it appeared that the *Pseudomonas* G1 inhibited the ability of *Rhodococcus* S1 to degrade anthracene and inhibited the ability of *Rhodococcus* S1 to grow. On the other hand, the presence of *Rhodococcus* S1 positively affected the growth of *Pseudomonas* G1 when anthracene was the only carbon and energy source. This conclusion was reached because *Pseudomonas* G1 was capable of growth in medium where anthracene was the only growth substrate when in the presence of *Rhodococcus* S1 but not in its absence.

3.5 Mixed Substrate Studies

Figure 3.14 revealed that anthracene was degraded faster in the coculture than in the pure culture of either microorganism: this coculture was established from *Rhodococcus* S1 growing on anthracene but *Pseudomonas* G1 from glycerol stock. In the second situation where initial cell numbers of *Rhodococcus* S1 were 20x greater than *Pseudomonas* G1 cell numbers, anthracene degradation of the coculture was similar to the pure culture of *Rhodococcus* S1 (Fig. 3.15). Subsequent studies with established cocultures showed that *Pseudomonas* G1 actually inhibited the ability of *Rhodococcus* S1 to degrade anthracene (Fig. 3.16). Therefore some other factor must have been positively affecting this coculture in Figure 3.14. It was suspected that the easily utilizable glycerol from the *Pseudomonas* G1 stock may have caused this result.

Likewise, the reason that *Pseudomonas* G1 was positively affected by *Rhodococcus* S1 was an interesting question. Did *Rhodococcus* S1 provide a carbon source to *Pseudomonas* G1 from the metabolism of anthracene? While trying to answer

this question it was discovered by Tongpim (1997) that *Rhodococcus* S1 could cometabolize other PAHs in the presence of anthracene . The anthracene used in these experiments was 99% pure and the 1% contamination was confirmed to be phenanthrene. So it was thought that *Pseudomonas* G1 may have been able to degrade phenanthrene or that *Rhodococcus* S1 may have cometabolized the phenanthrene thereby providing a phenanthrene metabolite as a possible carbon source for *Pseudomonas* G1.

3.5.1 The effect of a simple carbon source on the degradation of anthracene by pure cultures

Substantial quantites of glycerol were found to have been added to the mixed culture in Figure 3.14 when determining the interaction between *Rhodococcus* S1 and *Pseudomonas* G1 so that carbon sources were 0.05% anthracene and 0.01% glycerol. Therefore, to determine the effect of glycerol on the ability of each isolate to degrade anthracene, glycerol was added at the same concentration to pure cultures of *Pseudomonas* G1 and *Rhodococcus* S1 in Figures 3.17 and 3.18, respectively. Glycerol did not affect the rate or extent of removal of anthracene by *Pseudomonas* G1 after 14 and 21 days (Fig. 3.17). While *Pseudomonas* G1 was able to utilize glycerol as revealed by an increase in turbidity of the sample, *Pseudomonas* G1 could not metabolize or cometabolize anthracene in the presence of glycerol.

On the other hand, the ability of *Rhodococcus* S1 to degrade anthracene was enhanced upon the addition of glycerol (Fig. 3.18). The lag time before degradation occurred also appeared to decrease. Therefore the increased extent of degradation of anthracene in the mixed culture in Figure 3.14 can be attributed mainly to the addition of glycerol to the medium which stimulated the growth and degradation rate of *Rhodococcus* S1 only.

Figure 3.17 The effect of an alternate simple carbon source on the potential of *Pseudomonas* G1 to degrade anthracene.

The capacity of *Pseudomonas* G1 to degrade anthracene over 14 or 21 days was monitored in the presence of glycerol (solid gray bar) and in the absence of glycerol (hatched bar). Each sample was extracted in triplicate and error bars represent the standard deviation of each sample. Each sample is represented as the proportion of anthracene in the roller bottle when compared to the sterile control (black column).

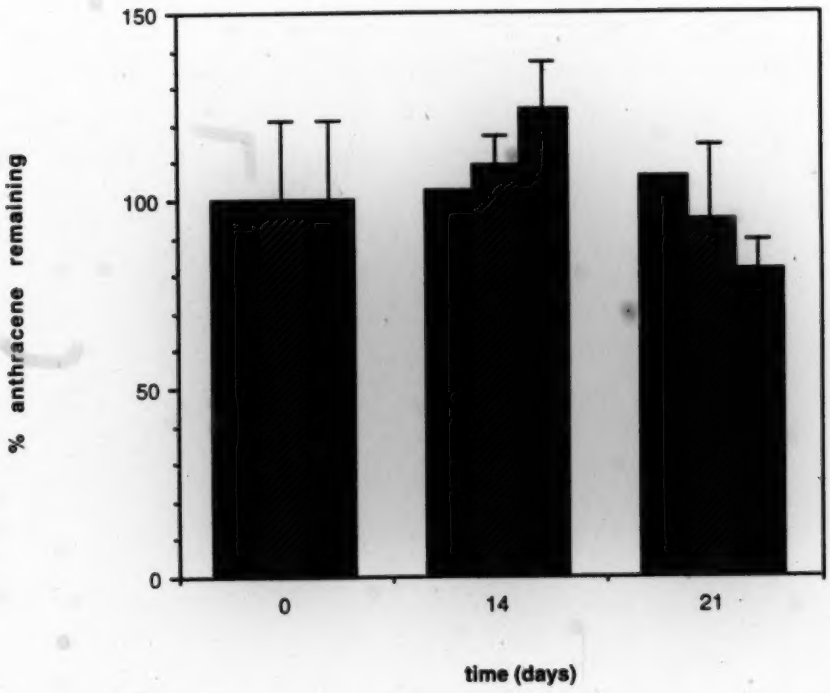
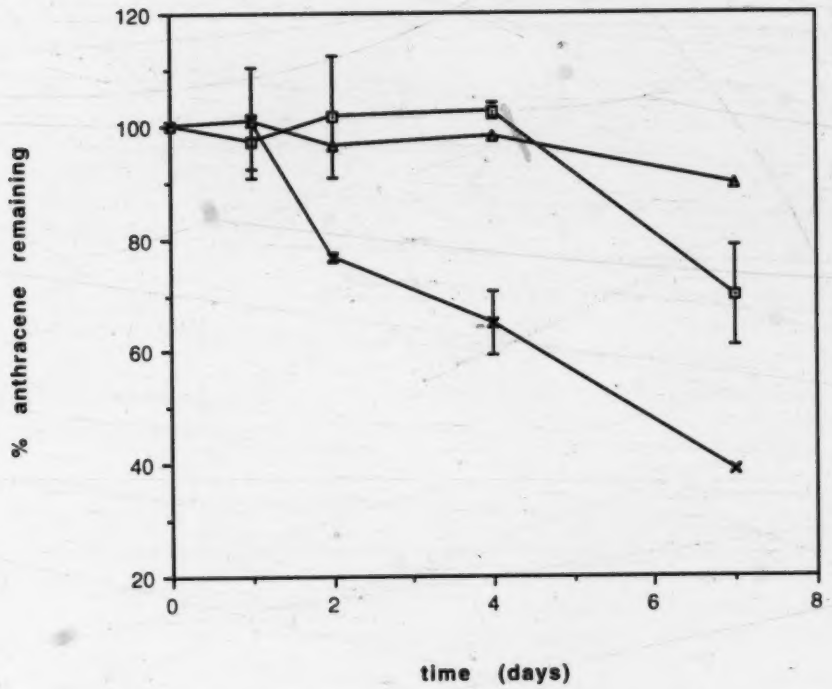

150
100
50
0
% anthracene remaining
0
14
21
time (days)

Figure 3.18 The effect of glycerol on the degradation rate of anthracene by *Rhodococcus* S1.

The proportion of anthracene degraded by *Rhodococcus* S1 was monitored in roller bottles to which glycerol was either added (x) or not (open squares). Duplicate roller bottles were used and each point represents the average of both duplicates with the error bars representing the standard deviation of each sampling point.


120
100
80
60
40
20
0
2
4
6
8
% anthracene remaining
time (days)

3.5.2 Degradation of alternate PAHs by the mixed culture

While it was known that the degradation of anthracene was negatively affected by this mixed culture, it has been suggested by other research that increasing the types of organisms present in a culture will also increase the enzymatic capabilities of that mixed culture. These enzymes may act together to enhance degradation of other similar compounds. This is believed to be the mechanism which occurs in mixed cultures that cometabolically degrade large PAHs.

3.5.2.1 Ability of each component of the coculture to utilize phenanthrene

The mixed culture, along with its pure culture components, was tested for growth on phenanthrene in the presence of anthracene (Fig. 3.19). Anthracene levels decreased more after 14 days in the pure culture of *Rhodococcus* S1 than the coculture. In the coculture and in the pure culture of *Rhodococcus* S1, phenanthrene levels also decreased. However, in the pure culture of *Pseudomonas* G1, phenanthrene levels did not decrease significantly. This decrease was due to the cometabolism of phenanthrene to its corresponding dihydrodiol by *Rhodococcus* S1 because neither *Rhodococcus* S1 nor *Pseudomonas* G1 was able to use phenanthrene as a source of carbon and energy but *Rhodococcus* S1 can cometabolise phenanthrene in the presence of anthracene (Tongpim, 1997). The possibility that *Pseudomonas* G1 used the cometabolite as a source of energy could not be definitely ascertained by this experiment. Therefore, phenanthrene was not a degradable compound for *Pseudomonas* G1 but its cometabolised dihydrodiol produced by *Rhodococcus* S1 may have served as a carbon source for *Pseudomonas* G1.

Figure 3.19 The ability of the coculture to degrade alternate PAHs

The stable mixed culture and its component pure cultures were incubated in the presence of glass beads coated with 0.05% anthracene and 0.05% phenanthrene in a roller tube. Black bars and hatched bars represent the capacity of *Pseudomonas* G1 to degrade the PAH at day 0 and after 14 days, respectively. The capacity of the pure culture of *Rhodococcus* S1 to degrade each PAH after 0 days (dark gray bar) and 14 days (white bar) was also compared to the degradative ability of the stable coculture of *Rhodococcus* S1 and *Pseudomonas* G1 after 0 (lined bar) and 14 days (light gray bar). Each sample was extracted in triplicate.

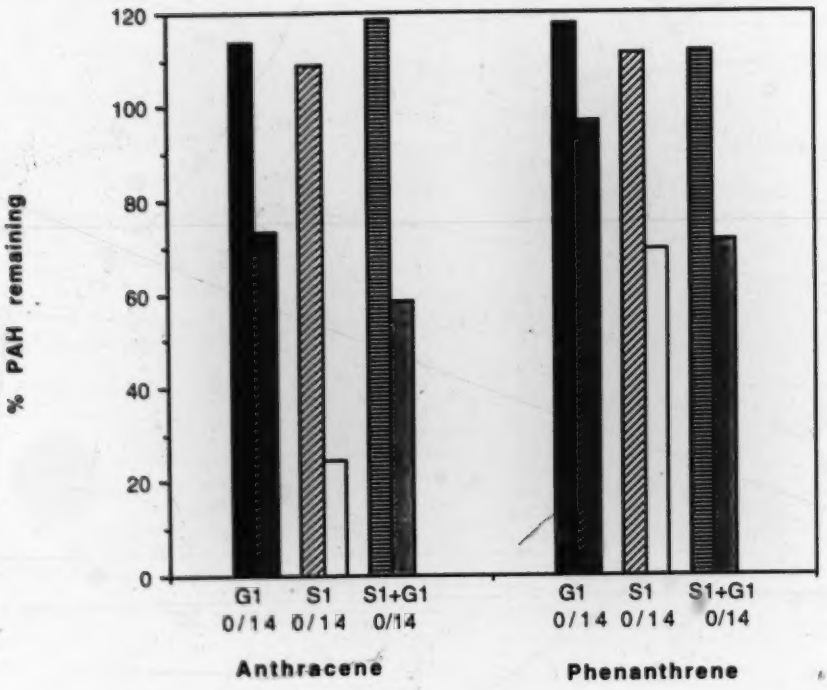
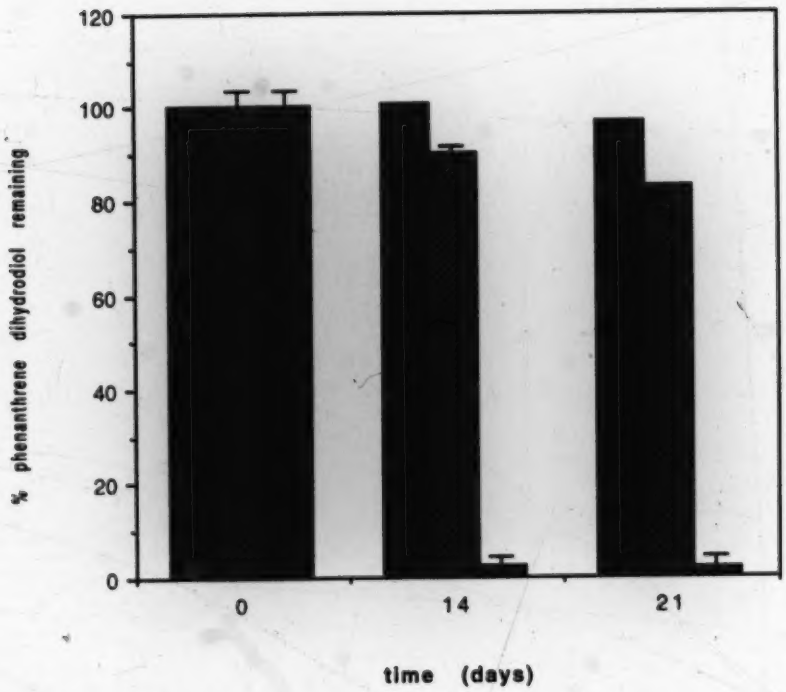

% PAH remaining
120
100
80
60
40
20
0
G1 0/14
S1 0/14
S1+G1 0/14
G1 0/14
S1 0/14
S1+G1 0/14
Anthracene
Phenanthrene

Figure 3.20 Degradation of a phenanthrene dihydrodiol by *Pseudomonas* G1. Phenanthrene dihydrodiol was prepared as described in the Materials and Methods. The degradation of the dihydrodiol after 14 and 21 days by *Pseudomonas* G1 (hatched bar) and the wildtype strain of *Pseudomonas fluorescens* LP6a (gray bar) was monitored by HPLC of the extracts (extraction efficiency of the sterile control is represented by the black bar).


120
100
80
60
40
20
0
% phenanthrene dihydrodiol remaining
0
14
21
time (days)

3.5.2.2 Degradation of a phenanthrene dihydrodiol by *Pseudomonas* G1

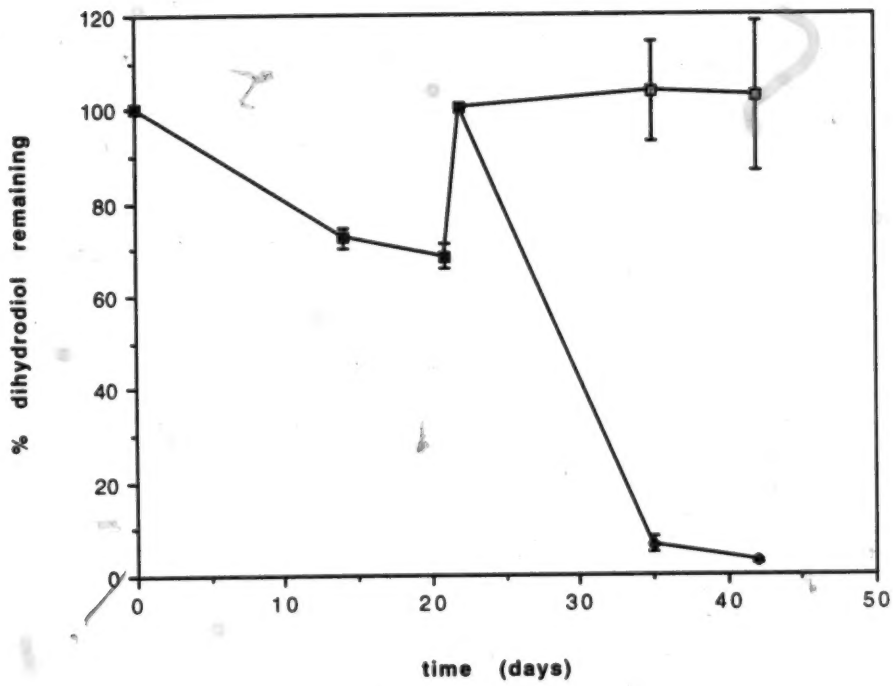
To determine if a phenanthrene dihydrodiol would be metabolised by *Pseudomonas* G1, a mixture of 1,2- and 3,4-phenanthrene dihydrodiols was prepared using a *Pseudomonas fluorescens* LP6a :: Tn5 mutant. This bacterium is blocked in metabolism beyond the production of phenanthrene dihydrodiol (Foght and Westlake, 1996). The culture was grown to a high optical density and induced by salicylate to produce its dioxygenase. The culture was added to a buffer containing phenanthrene and incubated for 3 days. It was centrifuged and filtered and the purity of the dihydrodiol in the supernatant was determined. The extract was 97-99% pure based on separation by HPLC, GC or TLC.

The dihydrodiol was added onto glass beads and G1 was inoculated into the sample. The extent of degradation was monitored after 14 or 21 days (Fig. 3.20). The wild type strain of *Pseudomonas fluorescens* LP6a completely degraded the phenanthrene dihydrodiol. The degradation of the dihydrodiol by *Pseudomonas* G1 was low.

Because of the low ability of *Pseudomonas* G1 to degrade the phenanthrene dihydrodiol, this transformation was suspected to be cometabolic. The transformation may require energy which the cell uses, but since there were no nutrients for growth, the transformation ceased when the stored energy in the cell was depleted. To test this hypothesis, *Pseudomonas* G1 was grown in the presence of the dihydrodiol for 21 days and degradation was monitored. After 21 days, *Pseudomonas* G1 was transferred either to a roller tube containing a simple carbon source (glycerol) and the dihydrodiol or to a tube containing only the dihydrodiol and depletion of this compound was monitored in both tests by HPLC (Fig. 3.21).

In the absence of glycerol, degradation of phenanthrene dihydrodiol did cease after 1 transfer. As well, the optical density of the medium did not increase over the entire

Figure 3.21 Cometabolism of the phenanthrene dihydrodiol by *Pseudomonas* G1. *Pseudomonas* G1 was incubated in the presence of a mixture of phenanthrene-1,2-dihydrodiol and phenanthrene-3,4-dihydrodiol in roller tubes containing 10 g glass beads and 10 g minimal medium for 21 days. After 21 days, the culture was transferred to a sterile roller tubes containing phenanthrene dihydrodiol with glycerol (solid triangles) as an alternate carbon source or without glycerol (open squares) for a further 21 days. All samples were prepared in triplicate and error bars represent the standard deviation of these samples.


% dihydrodiol remaining
120
100
80
60
40
20
0
0
10
20
30
40
50
time (days)

course of the experiment demonstrating the inability of *Pseudomonas* G1 to use this phenanthrene dihydrodiol as a carbon and energy source. When glycerol was added to the medium, the dihydrodiol was completely degraded after 14 days and optical density of the medium increased dramatically in the first two days.

These results revealed that the gram negative organism, *Pseudomonas* G1, possessed enzymes capable of cometabolizing an intermediate in the degradation pathway of phenanthrene by *Pseudomonas fluorescens* LP6a. However, the similarity of this dihydrodiol to the unknown dihydrodiol produced by *Rhodococcus* S1 was not determined.

4.0 DISCUSSION

Fast, economically feasible removal of PAHs by a stable mixed culture is sought by many bioremediative techniques especially those involving the use of bioreactors. The roller drum bioreactor developed by Masliyah et al. (1992) achieved rapid microbial degradation of the contaminant by providing an adequate oxygen supply to the community and the high proportion of soil in the slurry makes this process economically feasible. However, many of the interactions between components of the system in which bacterial degradation of the contaminant is enhanced are still not understood.

It has been proposed that, because of the low aqueous solubility, the rate of PAH degradation may be a function of mass transfer from the solid to aqueous phase (Volkering et al., 1992). Since anthracene was distributed over a greater surface area in the soil slurries than the crystal surfaces in liquid cultures, the rate of dissolution of anthracene should have been greater. Comparison of the anthracene-coated glass beads in the roller bottle to anthracene crystals in liquid medium in shake flasks confirmed the enhanced rate of dissolution of anthracene in the model soil system.

Besides the physical interactions of the bacteria and the contaminant as a growth substrate, there are undoubtedly interactions between various members of the bacterial community. Cometabolism, the production of surfactants to increase the solubility of the contaminant, nutritional interdependency or production of growth inhibiting substances may play prominent roles in the degradation by the soil community of anthropogenic contamination. Choosing two or three organisms to study at random from this mixed culture provided insight into the interactions enhancing or inhibiting the removal of anthracene. Alternatively, it may be that the removal of anthracene by pure cultures in a soil slurry bioreactor may be as efficient as that observed by mixed cultures.

This project set out to determine the importance of both the anthracene dissolution rates and bacterial interactions in the enriched soil community on the degradation of anthracene.

4.1 The effect of surface area on the dissolution rate of anthracene.

Since bacteria are suspected to degrade only the soluble fraction of PAHs (Wodzinski and Coyle, 1974) it was expected that the glass beads would provide a greater level of soluble anthracene in the aqueous medium than did liquid cultures. Stucki and Alexander (1987) have shown that the rate of degradation of PAHs is dependent upon the rate of dissolution of that PAH. Further, Volkering et al. (1992) and others (Thomas et al., 1986) have shown that the surface area of the hydrophobic contaminant exposed to the medium influences the rate of dissolution of that contaminant. Thus the increase in the dissolution rate of anthracene from glass beads may have been due to the increased surface area of anthracene on the glass beads or it may be due to the pulverization of the anthracene crystals which are on the glass beads, further increasing the surface area of anthracene particles. The amount of anthracene added to the sample may also have affected the dissolution rate because more anthracene added exposes a greater surface area of anthracene to the medium.

The results from this research appear to confirm the increased surface area hypothesis. The increased surface area of anthracene exposed to the medium was achieved by coating the glass beads with anthracene and this was compared to the addition of crystals with a minimal surface area. When an equal surface area of the anthracene was exposed to the medium by either coating the glass beads or the test tube, similar rates of dissolution were observed. The addition of a small crystal of anthracene with minimal surface area resulted in the slowest rate of dissolution of anthracene, confirming the importance of surface area in affecting the rate of dissolution of anthracene.

Thomas et al. (1986) have shown that the spontaneous dissolution rate of 4-chlorobiphenyl was dependent upon the surface area of this compound exposed to aqueous medium. They added the same mass of various sizes of 4-chlorobiphenyl (4-CB) particles to aqueous solution and found that the rate of solubilization increased with increasing

surface area. They also showed that the rate of solubilization of this compound initially exceeded the rate of growth and 4-CB accumulated in the medium. As cell numbers increased, the concentration of 4-CB in the medium decreased to undetectable levels.

It would have proven interesting to observe the aqueous solubility of anthracene in the presence of *Rhodococcus* S1 or *Pseudomonas* G1 or in the presence of sterile supernatants of these cultures. If a biosurfactant was produced, the sterile supernatant may have increased either the extent or rate of solubilization of anthracene. Other actinomycetes have been shown to produce trehalose lipids or neutral lipids which may increase the solubility of anthracene (Kosaric et al., 1993).

Research by Gray et al. (1994) with the original mixed culture from which *Rhodococcus* S1 was isolated, discovered that filter sterilized medium from the mixed culture after 7 days growth on anthracene increased the rate of solubilization of anthracene. Further research has discovered that this increase may be due to components produced by the mixed culture rather than extracts from the soil itself. However, the increase in the rate and extent of solubilization of anthracene by the spent medium was still very low and most anthracene was present in the solid phase.

4.1.1 The role of mechanical disruption on the dissolution of anthracene

Coating the glass beads with ^{14}C-anthracene was expected to give a higher rate of dissolution than coating the test tube wall because the glass beads grind against each other. As a result, the anthracene on the glass beads would have been pulverized into smaller particles thereby increasing the surface area exposed to the medium. Since the rates of dissolution of anthracene coated on glass beads was similar to samples not exposed to pulverization (only test tube wall coated), grinding of the anthracene particles appeared to play only a minor role in affecting the spontaneous dissolution of anthracene.

However, when uncoated glass beads were added to a test tube coated with ^{14}C-anthracene, the rates of dissolution were significantly higher than the other conditions. It appeared that the quality or nature of the surface that was coated with anthracene may also have had a significant effect. The surface of the test tube that is coated with anthracene may have been smoother than the glass beads. As a result, the anthracene may be more easily sloughed off and pulverization of the crystals by the glass beads may play a more important role in increasing the rate of dissolution of anthracene. When the glass beads were labeled with ^{14}C-anthracene, they were coated onto cracks and fissures in the glass beads as well. These crystals would not be exposed to the friction from other glass beads rolling against each other and therefore the surface area exposed to the medium was not increased as dramatically.

4.1.2 The effect of concentration on anthracene solubility

The effects of the initial concentration of ^{14}C-anthracene coated onto the glass beads or the test tube wall was also determined. In these experiments, an equal surface area was labeled with two different concentrations of radioactive anthracene. Since surface area was the only factor expected to affect solubility of anthracene, an increase in concentration of anthracene added would have had no effect on the dissolution rate of anthracene.

However, the results from these experiments show that the rate of solubilization was dependent upon the initial concentration of anthracene added to the samples. For example, the rate of dissolution of anthracene where the test tube was labelled with 350 µg/L (3.5 µg added/10 mL)was greater than the overall rate when the test tube was labeled with 150 µg/L (1.5 µL/10 mL; compare Figs 3.3a and b). After 250 h, the larger concentration of anthracene added had reached a solubility limit of 13 µg/L whereas the lower concentration of anthracene reached a limit of 7.5 µg/L.

On initial examination, these results appear to contradict the literature. Volkering et al. (1992) has shown that mass transfer of hydrophobic compounds from the solid to liquid

phase is rate limiting and therefore, the initial concentration added should not affect the rate of dissolution. It was further shown by Volkering et al. (1993) that the rate of spontaneous dissolution was dependent only on the surface area exposed to the medium.

Since the same surface area was exposed in both situations, rates of dissolution of anthracene should have been the same. However, it may have been possible that 1.50 µg of anthracene (2 times the saturation limit in 10 mL water) may not have uniformly labeled the entire surface of the test tube or glass beads. If this were the case, then 3.5 µg anthracene (5 times the saturation limit) labeled onto these surfaces would have a greater surface area and thus a greater rate of dissolution.

To confirm that the entire surface area was not covered, one could add increasing concentrations of ^{14}C-anthracene to the same surface area of the test tube. If the rate of dissolution was dependent solely on the surface area as demonstrated by Thomas et al. (1986), then increasing concentrations of anthracene would result in increasing rates of dissolution until the entire surface was uniformly coated and a maximum of anthracene is exposed to the medium. At this point, further increases in anthracene concentrations should no longer affect the dissolution rate.

Additionally, from the SEM micrographs it was observed that the surface of the glass beads was unevenly coated. Uneven coating of the glass bead surface may depend on the rate of evaporation and more careful solvent removal from the glass beads might overcome this problem.

In all the radioactive experiments, the concentration of anthracene never approached the theoretical saturation limit of anthracene in water determined by May et al. (1978). Theoretical saturation limits of anthracene were determined to be between 40-70 µg/L. The results obtained from these experiments were consistent with those found by Gray et al. (1994). Fresh salts medium only achieved 20% of the expected solubility of 45 µg/L after 7 days. The results presented here found that solubility of anthracene within the salt medium increased even less than this after 10 days in samples containing the lowest

concentrations of anthracene. This was probably due to a lower surface area of anthracene exposed to the medium than in the experiment reported by Gray et al. (1994). Further, Klevens (1950) observed that some hydrophobic compounds may take several months to reach theoretical solubility limits.

Also, theoretical solubilities of anthracene may never have been reached because the solubility of a neutral organic compound decreases in solutions with increasing ionic strength (Sharp et al., 1989). Since this experiment was performed in minimal salts medium, the actual solubility of anthracene would be lower than theoretically predicted in water.

4.2 Growth of *Rhodococcus* S1 in roller bottles

Thomas et al. (1986) suggested that bacteria may use insoluble carbon sources in three ways: (i) biologically enhancing the rate of solubilization of the insoluble carbon source, (ii) degrading it as it spontaneously dissolves in solution or (iii) by mechanisms involving physical contact with the insoluble phase of the substrate. When *Rhodococcus* S1 was initially incubated in the roller bottle environment, a 4 - 5 day lag time was observed before significant levels of anthracene were degraded. So it was expected that at least one of the three factors listed above caused this lag time. Tongpim (1997) has found that there is a two day lag before cell numbers increase when *Rhodococcus* was grown in liquid culture.

Originally, cells were transferred from a shake flask to the roller bottle environment as a 5% inoculum. Therefore, if a solubilizing agent was formed, it would also have been transferred into the roller bottle and this lag still occurred. Unless a certain concentration of the solubilizing agent was required before anthracene solubility increased, the agent should not affect the lag time observed by these cultures in the roller bottle.

To determine if *Rhodococcus* S1 mainly used anthracene that was spontaneously dissolved into solution, the roller bottle was preincubated for 5 days to achieve a higher

concentration of anthracene in solution before inoculation with *Rhodococcus* S1. It was proposed that the initial concentration of anthracene found in the medium may not have been sufficient to allow growth of *Rhodococcus* S1. The concentration may have been too low to induce genes for the production of the appropriate enzymes. Alternatively, enzymes used for metabolizing the anthracene may have required higher concentrations than the initial concentrations of anthracene in solution in order to be active. As anthracene levels approach those equivalent to the affinity for *Rhodococcus* S1 to take up anthracene, degradation of anthracene may have begun to occur. In these experiments, the lag time decreased to 3 days. Therefore some anthracene had dissolved and could be used faster than previous tests. However, a lag time of three days before anthracene degradation occurred showed that spontaneously dissolved anthracene was probably not the predominant form of anthracene used by*Rhodococcus* S1. This suggests that some time may be required for *Rhodococcus* S1 to come in physical contact with anthracene crystals. Further evidence against the idea that *Rhodococcus* S1 used only soluble anthracene was observed in the degradation curve of *Rhodococcus* S1. After a 4 - 5 day lag time, levels of degradation occurred rapidly. If *Rhodococcus* S1 could degrade only dissolved anthracene, rates of degradation would have been linear throughout the entire incubation.

Scanning electron micrographs showed that *Rhodococcus* S1 accumulated on the glass beads where anthracene was expected to accumulate and that *Rhodococcus* S1 bound to the surface of anthracene crystals. Where *Rhodococcus* S1 bound to the surface, indentations were formed in the crytals. This would further suggest that the mechanism of degradation of anthracene appeared to require physical contact with the insoluble substrate. The cell wall of *Rhodococcus* sp. possesses extremely hydrophobic components such as mycolic acids (Goodfellow, 1992). These lipophilic compounds may enhance the ability of *Rhodococcus* S1 to attach to anthracene crystals and other hydrophobic constituents in soil which S1 may use as a carbon and energy source. Therefore, it was proposed that attachment of *Rhodococcus* S1 to its insoluble carbon source is the most likely reason that a

lag time occurred. The lag time was not as evident in liquid cultures because the coil may have helped remove the cells from the anthracene crystals thus preventing colonization of *Rhodococcus* S1 to the anthracene crystal.

During this colonization period in roller bottle systems, cell densities increased by 400 fold. But after the colonization time, cell densities only appeared to increase 5 fold (Fig. 3.5). Since anthracene was the only source of carbon and energy, cell densities were expected to increase more after the lag time than before the lag time because anthracene was degraded at a faster rate after the lag time. Therefore it was possible that the initial burst in the growth of *Rhodococcus* S1 may have been due to growth of this organism on the fixed carbon of dead cells.

The small increase in cell densities after colonizing the anthracene crystal may have been due to clumping of the cells together onto the anthracene crystals. A problem with viable counts is that clumped cells grow as one colony on PCA and therefore actual cell densities are much higher. Thus, one anthracene crystal may have provided growth for several organisms but only appeared as one organism by the viable count procedure. A more accurate estimate of the growth curve may be achieved through the estimation of total protein in the sample.

It has been predicted that actinomycetes, such as *Rhodococcus* S1, play a very important role in the degradation of humus and other insoluble compounds in the soil (Kästner et al., 1994). These organisms attach to hydrophobic soil surfaces and cleave constituents off humic substances. It is also possible that the enzymes present in the cell membrane may also serve to attach the cell to the anthracene crystal by forming a complex with the crystal form of anthracene (Burns, 1979). However, the formation of this enzyme-substrate complex and the ability of these organisms to take up the insoluble substrate are not completely understood.

These results may also explain why acclimated cultures of *Rhodococcus* S1 in the roller bottle did not exhibit a significant lag time before anthracene degradation occurred

(Fig. 3.9). The cells that were transferred were able to degrade anthracene at a faster initial rate; however, the extent of degradation was the same. Since *Rhodococcus* S1 was transferred from a roller bottle containing anthracene coated glass beads, the initial degradation rates may have been due to any cells that colonized the anthracene crystals from the previous transfer. After 4 days, the culture that was not attached to anthracene crystals or had been sloughed off the glass beads had time to colonize and degrade other crystals.

4.3 Mixed culture studies

The original mixed culture enriched for growth on anthracene was able to degrade anthracene from loaded soil at a rate of up to 300 mg·kg soil^{-1}·day^{-1}. This mixed culture was transferred to a roller bottle containing anthracene coated glass beads. The anthracene removal rates by this mixed culture had decreased to 50 mg·kg glass beads^{-1}·day^{-1}.

These results show that factors present in the soil but not in the glass beads enhanced the rate of degradation of anthracene. Organic matter and detritus in the soil may have provided other carbon sources to members of the soil community which may have stimulated degradation. The soil also had a greater surface area for attachment of bacteria and anthracene (Gray et al., 1994). The attachment of microorganisms to soil surfaces may enhance their degradative capacity by bringing them in close association with a carbon source and nutrients (Burns, 1979).

Microorganisms from the original mixed culture were isolated to determine if pure cultures could degrade anthracene as well as the mixed culture. The degradation rates of anthracene by the mixed culture were compared to the rates of two isolates from this mixed culture. One of the isolates, *Pseudomonas* G1, was unable to metabolise anthracene as a sole carbon and energy source. The other isolate, *Rhodococcus* S1, removed anthracene from the glass beads at a rate of 25 mg·kg glass beads^{-1}·day^{-1}. However, the growth rate of *Rhodococcus* S1 was significantly higher in the roller bottle environment when compared to the rates reported by Tongpim and Pickard (1996) in liquid culture.

These results showed that interactions in the mixed culture may have enhanced the removal of anthracene from the roller bottle because the mixed culture removed anthracene at a faster rate than either pure culture. Therefore, the provision of nutrients, cometabolism or the production of surface active agents may be important factors in enhancing the rate of degradation of anthracene in this mixed culture.

4.3.1 Interactions between two slow growing bacteria

Pure culture Gram positive bacterium, A1 was isolated from the same original creosote contaminated soil that both *Rhodococcus* S1 and *Pseudomonas* G1 were isolated from. However, A1 was not found in the mixed culture enriched on anthracene. This suggested that it was competed out of the mixed culture or was present at very low concentrations. Isolate A1 was able to grow on phenanthrene, pyrene and naphthalene as well as anthracene. A coculture of A1 with *Rhodococcus* S1 was as effective at removing anthracene as either of the pure cultures. Total viable counts were also similar in pure and mixed cultures. Since each organism achieved an overall lower maximal density than in pure cultures, the interaction was considered competitive. This interaction was considered a resource based competition because both organisms competed for the same carbon and energy source (Atlas and Bartha, 1993). *Rhodococcus* S1 was able to grow more efficiently than A1 when anthracene served as a carbon source; thus, *Rhodococcus* S1 could outcompete A1 for anthracene.

Competition can be limited if both populations can avoid directly competing by using different resources (Fredrickson and Stephanopoulos, 1981). This may be how A1 survives in creosote contaminated soil but not in soil where anthracene is the only substrate. While anthracene may be a significant component of creosote, approximately 17 other compounds are present at concentrations >1% in creosote (Mueller et al., 1989). As A1 was capable of using at least 3 other PAHs from creosote contaminated soil as sole carbon and energy sources, the presence of other PAHs may have decreased the extent of

this competition between A1 and S1. Studies following the interactions of A1 with other members of a creosote enrichment culture may have also been interesting.

Caution should be taken when determining this interaction as a competition based solely on viable counts. The viable count procedure may not be accurate in determining cell numbers in soil systems. Clumping of the bacteria and attachment of the bacteria to the soil are among reasons which may result in an artificially low viable count for either organism.

4.3.2 Interactions between *Pseudomonas* G1 and *Rhodococcus* S1

Since the original mixed culture consisted of two morphologically distinct colony types, a coculture consisting of one member from each subpopulation was established. When *Rhodococcus* S1 outnumbered *Pseudomonas* G1 by 20 fold, the rates of anthracene degradation were equivalent to rates observed in pure cultures of *Rhodococcus* S1 and growth of *Rhodococcus* S1 in the cocultures was slightly inhibited when compared to the pure culture. On the other hand the final levels of *Pseudomonas* G1 in the medium was similar to the cell concentrations of *Rhodococcus* S1. It may have proven interesting to follow this coculture after one transfer to determine the effect of this change in cell ratios on the degradation rate of anthracene and growth rate of each member of the coculture.

To establish a coculture of *Pseudomonas* G1 and *Rhodococcus* S1, equal cell concentrations of each pure culture were added together in liquid culture. After three monthly subcultures the coculture was arbitrarily chosen as an established community. In the established coculture, the rate of anthracene degradation and growth rate of *Rhodococcus* S1 was lower than pure cultures of *Rhodococcus* S1.

By comparison of the growth of the two pure cultures and their coculture it was determined that the growth of *Rhodococcus* S1 was inhibited by *Pseudomonas* G1. However, the growth of *Pseudomonas* G1 was stimulated by *Rhodococcus* S1 because *Pseudomonas* G1 required the presence of S1 in order to grow in the presence of

anthracene. Also, *Pseudomonas* G1 became the numerically dominant microorganism present in the stable coculture while the viable counts of *Rhodococcus* S1 decreased significantly.

Therefore, according to the interactions described previously in the Introduction, this relationship would appear to be a predator/parasitic relationship. This parasitic relationship may have been a metabolic type of parasitism rather than a direct physical parasitism. Since *Pseudomonas* G1 was numerically dominant, it must have a more efficient metabolism. It was unable to utilize anthracene and the anthracene added was 99% pure. Therefore, *Pseudomonas* G1 may have used metabolites normally utilized by *Rhodococcus* S1. Some of the initial reactions involved in the metabolism of PAHs require energy. It was possible that *Rhodococcus* S1 expended this energy to activate the metabolism of anthracene and that *Pseudomonas* G1 did grow in close proximity to *Rhodococcus* S1 and utilized the oxidized anthracene as its own metabolite. Or perhaps *Pseudomonas* G1 parasitized *Rhodococcus* S1 by using nutrients produced by *Rhodococcus* S1 and required by both organisms in the coculture. Since *Pseudomonas* G1 was a faster grower, it effectively outcompeted *Rhodococcus* S1 for substrates.

This may be difficult to confirm since metabolites of anthracene degradation by *Rhodococcus* S1 have not been found. However, within the vicinity of *Rhodococcus* S1 on the anthracene crystal, low concentrations of metabolites may have been present. If *Pseudomonas* G1 grows in close proximity to *Rhodococcus* S1, *Pseudomonas* G1 would have been able to scavenge these metabolites.

The addition of a simple carbon source, such as glycerol, provided a simple source of energy to both *Rhodococcus* S1 and *Pseudomonas* G1 and therefore the parasitic relationship appeared to be alleviated. *Rhodococcus* S1 could use the glycerol for energy and growth. The increased cell numbers resulted in increased rates of metabolism of anthracene. These increased rates of metabolism were similar to rates of degradation

observed by the mixed culture. At the same time, *Pseudomonas* G1 was also able to use the glycerol and did not require the interaction with S1 in order to survive.

Clearly, *Pseudomonas* G1 did not produce a growth inhibiting substance against *Rhodococcus* S1, a possible alternative. If this were the case, continuous transfers of this coculture to subsequent flasks containing only anthracene as the sole source of carbon and energy source would have resulted in eventual death to the coculture because *Rhodococcus* S1 would eventually become diluted out. Once *Rhodococcus* S1 became diluted out, *Pseudomonas* G1 would eventually die because it was unable to degrade anthracene. Even after 15 monthly transfers, this did not occur.

The negative interaction seen in this coculture did not affect the degradation of anthracene in the original mixed culture reported by Gray et al. (1994) and Bannerjee et al. (1995) for several reasons. First, *Rhodococcus* S1 or *Pseudomonas* G1 may have been able to utilize alternate carbon sources in the original soil. This would have alleviated the need for *Pseudomonas* G1 to depend on *Rhodococcus* S1 for a carbon source. Second, interactions of these two organisms may have been less important when compared to the interactions of *Rhodococcus* S1 and *Pseudomonas* G1 with other organisms in the original mixed culture. For example, other organisms in the mixed culture may have produced growth inhibiting compounds to reduce the growth rate of the fast growing population.

Third, these organisms may have limited their interaction due to a different spatial arrangement in the original soil. Stringfellow and Aitken (1994) found that two dissimilar pseudomonads thrived in different niches when incubated on phenanthrene in liquid culture. One isolate grew as a biofilm on phenanthrene crystals while the other degraded phenanthrene in solution only. Therefore any interactions between these organisms were minimized due to physical separation of these two isolates.

The glass beads may not have allowed an adequate surface for attachment by *Rhodococcus* S1. The charged and smooth surface of the glass beads may have weakened the ability of *Rhodococcus* S1 to attach to that surface. *Rhodococcus* S1 has a very

hydrophobic cell wall which may have encouraged attachment to the original soil matrix. The cell wall of most *Pseudomonas* sp. is usually not as hydrophobic as *Rhodococcus* because *Pseudomonas* has no mycolate in its cell wall. Therefore, each organism of this coculture occupied separate niches in the original soil. *Rhodococcus* S1 would have attached to some soil surfaces while *Pseudomonas* G1 would have attached to a different portion of the soil matrix or been present in the soil pore water. This would have alleviated the interaction somewhat. On the smooth surface of the glass beads, *Rhodococcus* S1 was easily sloughed off, possibly forcing an interaction with *Pseudomonas* G1 in aqueous medium.

Finally, *Rhodococcus* S1 and *Pseudomonas* G1 may not have been the organisms primarily responsible for PAH degradation in this soil. They were chosen because they appeared to be the dominant populations in the soil when grown on complex medium.

Other tests which could have been used to monitor the presence of *Rhodococcus* S1 or *Pseudomonas* G1 in the enrichment culture may have involved the use of phospholipid fatty acid analysis (PLFA). The PLFA profile of each organism is unique at a constant temperature. Some microorganisms possess unique phospholipids in their membranes which serve as a biomarker of the presence of that organism in the environment. By monitoring a unique lipid biomarker in the membrane of *Rhodococcus* S1, its presence in the stable coculture and the original mixed culture could be confirmed. One could follow this biomarker to determine if *Rhodococcus* S1 prefers to attach to the glass beads, soil, organic matter or to stay in the aqueous medium. Similar tests could also be performed on *Pseudomonas* G1. A procedure to quantify the presence of *Rhodococcus* S1 by following the lipid biomarker would also be beneficial in these studies. However, this procedure can be very time consuming and could not be carried out as routinely as GC analysis of substrate removal or metabolite production.

4.4 Mixed substrate interactions

Mixed cultures usually have a greater degradative capacity than pure cultures alone. Even though the interaction between *Pseudomonas* G1 and *Rhodococcus* S1 decreased the degradation rate of anthracene compared to pure cultures of *Rhodococcus* S1, it was expected that these organisms together may degrade other PAHs. Mueller et al. (1989) found that a mixed culture of 7 morphologically distinct organisms was capable of degrading 13 of 17 PAHs to undetectable levels within 3 days of inoculation into shake flasks. A single isolate from this community was capable of growth on only 5 of these PAHs (Mueller et al., 1990). Therefore, the other PAHs were degraded by other members of the community or by interactions within the community.

Thus, the ability of the coculture to degrade other PAHs was tested. It was proposed by Tongpim (1997) that *Rhodococcus* S1 possesses a cytochrome P-450 monooxygenase capable of cometabolising other PAHs. It was suspected that *Pseudomonas* G1 may also cometabolise other PAHs or utilize the products of PAH metabolism by *Rhodococcus* S1. *Pseudomonas* G1 could not degrade phenanthrene (fig. 3.19) but *Rhodococcus* S1 was able to cometabolise phenanthrene (Tongpim, 1997). A phenanthrene dihydrodiol produced by *Pseudomonas fluorescens* LP6a::Tn5 was purified and growth of *Pseudomonas* G1 on this substrate was followed. *Pseudomonas* G1 was able to cometabolise this phenanthrene dihydrodiol but not able to use it as a sole carbon and energy source.

Therefore, *Pseudomonas* G1 possesses enzymes capable of cometabolising this phenanthrene dihydrodiol. *Rhodococcus* S1 produces phenanthrene-9,10-dihydrodiol by cometabolism (Tongpim, 1997) while that produced by *Pseudomonas fluorescens* LP6a is a combination of the 1,2 and 3,4 isomers of phenanthrene dihydrodiol (Foght and Westlake, 1996). Consequently, the enzymes possessed by *Pseudomonas* G1 may be able to convert the dihydrodiols, produced by *Rhodococcus* S1, into metabolites which can be used as a carbon and energy source. Thus the phenanthrene dihydrodiol tested in these

experiments may not accurately reflect the ability of *Pseudomonas* G1 to grow in coculture with *Rhodococcus* S1.

To confirm this hypothesis, *Pseudomonas* G1 should be grown in the presence of the phenanthrene and anthracene dihydrodiols produced by *Rhodococcus* S1. The phenanthrene dihydrodiol, however, has been difficult to purify in quantifiable amounts and metabolites of anthracene metabolism have not yet been found for *Rhodococcus* S1. It is suspected that the rate limiting reaction for the growth of *Rhodococcus* S1 on anthracene is the conversion of anthracene to anthracene dihydrodiol which is then quickly utilized by *Rhodococcus* S1. Due to the numerical dominance of Pseudomonas G1 in the coculture, it must be using metabolites produced by *Rhodococcus* S1. If these compounds can be purified and the metabolism of them can be demonstrated, the interaction that exists between these two bacteria may be further confirmed.

4.5 Conclusions

The objectives of this thesis were reasonably achieved. It was proposed that the mass transfer rates of anthracene in soil was greater than in liquid cultures and that the original mixed culture had a greater degradative capacity than the pure culture. The use of glass beads was effective at monitoring these factors. The mass transfer rates of anthracene increased with increasing surface area exposed to the aqueous medium. Also, the quality of the surface that was coated with anthracene affected the dissolution rate.

The rates of degradation of anthracene by a pure culture of *Rhodococcus* S1 were increased in the roller bottle when compared to liquid cultures. However, *Rhodococcus* S1 exhibited a lag time of 4 days in the roller bottle. This rate of degradation was not solely dependent upon the spontaneous dissolution rate of anthracene into the aqueous medium. Rather, it appeared that Rhodococcus S1 may degrade the solid substrate by either direct interaction or solubilizing the area around the crystal. It was possible that *Rhodococcus* S1 released a solubilizing agent directly into the medium to increase the overall solubility of

anthracene. But, the effect of this agent was not the cause for a lag time exhibited by *Rhodococcus* S1. However, more studies are needed to resolve these hypotheses.

Interaction of *Rhodococcus* S1 with a slow growing isolate from the same contaminated soil, A1, proved to be competitive. Isolate A1 was not found in the anthracene enrichment culture and may have been competed out during the enrichment process. However, isolate A1 was able to metabolise other PAHs found in creosote contaminated soil which may have alleviated the competition for anthracene in the original contaminated soil.

It was found that a fast growing isolate from the mixed culture negatively affected the growth and degradation rate of anthracene by *Rhodococcus* S1. This isolate, *Pseudomonas* G1, could not grow in the presence of anthracene as a sole carbon and energy source. But it could grow under these conditions in the presence *Rhodococcus* S1. In the presence of *Pseudomonas* G1, *Rhodococcus* S1 was outnumbered by 25:1. This suggested that *Pseudomonas* G1 was utilizing substrates produced by *Rhodococcus* S1. This relationship was predicted to be a metabolic parasitism. It was also suspected that *Pseudomonas* G1 may have been able to use PAHs that were cometabolised by *Rhodococcus* S1. The addition of a simple carbon source was able to alleviate this negative interaction.

Since it was suspected that *Pseudomonas* G1 used either intermediates of anthracene metabolism from *Rhodococcus* S1 or cometabolised phenanthrene, growth of *Pseudomonas* G1 in the presence of known metabolites of phenanthrene metabolism by other microorganisms was determined. *Pseudomonas* G1 was found to cometabolise these substrates showing that it possessed enzymes capable of degrading PAH metabolites. Isolation and purification of phenanthrene and anthracene metabolites from *Rhodococcus* S1 should be performed in order to confirm that *Pseudomonas* G1 is able to use these products as sole energy and carbon sources and thereby confirm that it negatively interacts with *Rhodococcus* S1 by competing for metabolites.

The glass beads served as a model soil to study interactions between bacteria in the roller drum bioreactor. However, the glass beads did not bind anthracene very effectively. The interactions of the bacteria and anthracene with the glass beads appear not to be as complex as in clay soils or sandy soils with high organic content. As a result, these studies help to confirm interactions between microorganisms within this bioreactor. However, any interactions that are determined should be extrapolated to real soils with a high degree of caution.

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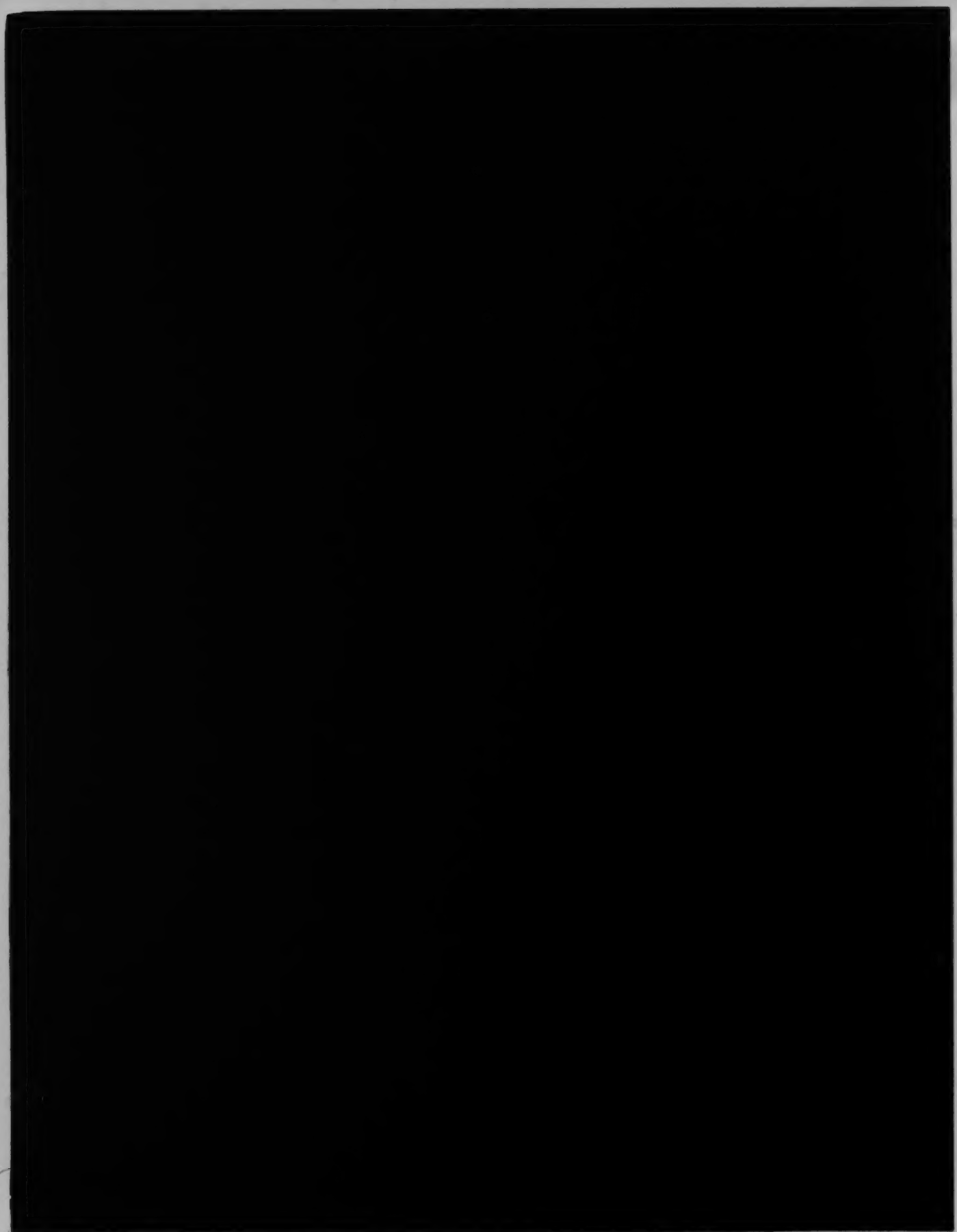
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